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Physical studies of adenylosuccinate synthetase

Michael Brian Bass
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PHYSICAL STUDIES OF ADENYLOSUCCINATE SYNTHETASE

Iowa State University PH.D. 1987

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Physical studies of adenylosuccinate synthetase

by

Michael Brian Bass

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

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GENERAL INTRODUCTION

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP forming), EC 6.3.4.4] catalyzes the first step in the synthesis of AMP from IMP. This reaction,

GTP + IMP + L-aspartate $\rightleftharpoons$ Adenylosuccinate + GDP + P_i

occupies a crucial regulatory point in the purine de novo biosynthetic pathway (1). The mechanism of the reaction has been the subject of many studies. In 1956, Lieberman (2) proposed the reaction proceeded via a 6-phosphoryl-IMP intermediate where the 6-oxygen of IMP makes a nucleophilic attack on the γ-phosphorus of GTP. In the next step, the α-nitrogen of aspartate attacks the 6-carbon of IMP leading to a tetrahedral adduct. Subsequently, adenylosuccinate is formed with the departure of inorganic phosphate. This mechanism was based on the finding that isotopically labelled oxygen in the 6-position of IMP is quantitatively transferred to inorganic phosphate (2). Fromm came to the same conclusion regarding the reaction mechanism in 1958 based on isotope exchange studies (3). In 1962, Miller and Buchanan (4) proposed a concerted reaction mechanism for adenylosuccinate synthetase. In this mechanism, all three substrates react simultaneously to form adenylosuccinate, GDP, and P_i. In 1978, Markham and Reed (5) proposed a reaction mechanism based on presteady-state kinetics using GTPγS in which the α-nitrogen of aspartate attacks the 6-carbon of IMP first. Subsequently, the oxy-anion attacks the γ-phosphorus of GTP. Initial-rate kinetics from a variety of sources has shown that the kinetic mechanism is Random Ter Ter (6-10). Unfortunately the kinetic mechanism

did not shed any light on the chemical mechanism, other than to show that there is a requirement that all of the reactants must bind to the enzyme before any chemical reaction occurs.

Subsequently, several reports have been published attempting to elucidate the chemical mechanism of the adenylosuccinate synthetase reaction. Using the chiral probe [γ-^{16}O,^{17}O,^{18}O]GTPγS, Webb et al. (11) showed that the stereochemical course of the reaction proceeded with net inversion of configuration at the phosphate. This finding, although consistent with all of the proposed mechanisms, did eliminate the possibility of a phosphoryl-enzyme intermediate in the mechanism.

Section I of this dissertation addresses the problem of which reaction mechanism occurs in the adenylosuccinate synthetase reaction. By using the method of NMR spectroscopy to measure the exchange of isotope from the β-γ bridge position to the β nonbridge position of [γ-^{18}O]GTP. Positional isotope exchange was found to occur in the presence of either IMP or IMP and succinate. The exchange did not occur in the presence of aspartate. The positional isotope exchange reactions show that the reaction occurs via a 6-phosphoryl-IMP intermediate as originally proposed by Lieberman (2).

A major problem encountered when performing the nuclear magnetic resonance experiments of Section I is exchange broadening that occurs when a compound undergoes chemical exchange. Section II describes the problem and the method used to allow measurement of the positional isotope exchange used in Section I.

Physical studies of adenylosuccinate synthetase from Escherichia coli have been difficult to preform due to the low concentration of the enzyme

present in the cell and the difficulty in purifying the enzyme to homogeneity. Physical studies of adenylosuccinate synthetase from mammalian sources, which can be obtained in larger quantities, is limited by the solubility of the enzyme (1). Section III describes the cloning and overproduction of adenylosuccinate synthetase from E. coli using a runaway-replication plasmid vector. Overproduction of the enzyme allowed a new purification method to be employed that resulted in a large yield of adenylosuccinate synthetase.

A recent report attempted to show that rat skeletal muscle hexokinase was an allosteric enzyme (12). Section IV shows that the allosteric mechanism described by Gregoriou et al. (12) is inconsistent with a large body of kinetic data for this enzyme and is also inconsistent with some of the data from their own lab (13).

Explanation of dissertation format The dissertation is written in the alternate dissertation format. Each section represents a paper each of which has been submitted to a scholarly journal for publication.

ABBREVIATIONS

CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
DTT	DL-dithiothreitol
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
GTPγS	(γ-thiophosphate)guanosine 5'-triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high-performance liquid chromatography
IPTG	isopropyl β-D-thiogalactoside
KP_i	a buffer of potassium phosphate, 1 mM EDTA, and 1 mM β-mercaptoethanol at pH 7.0
Mes	2-[N-morpholino]ethanesulfonic acid
NMR	nuclear magnetic resonance
PMSF	phenylmethylsulfonylfluoride
Tris	tris(hydroxymethyl)aminomethane
TE	a buffer of Tris and 1 mM EDTA at pH 7.5
TEAB	a buffer of triethylammonium bicarbonate at pH 8.0

SECTION I: THE MECHANISM OF THE ADENYLOSUCCINATE SYNTHETASE REACTION AS STUDIED BY POSITIONAL ISOTOPE EXCHANGE

The Mechanism of the Adenylosuccinate Synthetase Reaction as Studied by Positional Isotope Exchange

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ABSTRACT

In an attempt to gain insight into the mechanism of the rat muscle adenylosuccinate synthetase reaction, experiments using the technique of positional isotope exchange (isotope scrambling) were undertaken. [γ-^{18}O]GTP was prepared and incubated with Mg^{2+} and the synthetase in the presence of various ligands. Positional isotope exchange occurred, as measured by nuclear magnetic resonance (NMR) spectroscopy, when IMP was present. In the absence of IMP, with or without aspartate or succinate, the [γ-^{18}O]GTP did not exhibit scrambling. These results suggest that the adenylosuccinate synthetase reaction involves the participation of 6-phosphoryl-IMP as an obligatory intermediate. On the basis of experiments carried out in our laboratory as well as in others, we believe that GDP remains bound to the enzyme until the product, adenylosuccinate, is formed. All products may then dissociate randomly from the enzyme. The positional isotope exchange experiments, along with initial-rate experiments carried out in our laboratory, serve to explain the lack of partial exchange reactions associated with the synthetase (Fromm, H. J. (1958) Biochim. Biophys. Acta 29, 255-262), as well as the net inversion of configuration when chiral thio-GTP is converted to thiophosphate (Webb, M. R., Reed, G. H., Cooper, B. F., and Rudolph, F. B. (1984) J. Biol. Chem. 259, 3044-3046).

INTRODUCTION

Adenylosuccinate synthetase (IMP:L-Aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the reaction:

GTP + IMP + L-Aspartate $\rightleftharpoons$ GDP + P_i + Adenylosuccinate.

Because this reaction is the first step in the biosynthesis of adenine nucleotides from IMP, the enzyme plays an important role in the regulation of purine nucleotide interconversion. For an extensive review of adenylosuccinate synthetase, see Stayton et al. (1).

To date, there have been three mechanisms proposed for the adenylosuccinate synthetase reaction. The earliest was suggested by Lieberman (2) and involves a 6-phosphoryl-IMP intermediate. The second, proposed by Miller and Buchanan (3), involves a concerted reaction in which all three substrates participate simultaneously. The third mechanism, proposed by Markham and Reed (4), has aspartate attacking the 6-carbon of IMP in the first step of the reaction.

Recently, Webb et al. (5), have followed the stereochemical course of the adenylosuccinate synthetase reaction using chiral [^{16}O,^{17}O,^{18}O]-thiophosphate in the γ position of GTP. They found that the reaction proceeds with net inversion of configuration. These results are consistent with all three mechanisms if one proposes direct phosphoryl transfer from the γ position of GTP to the 6-oxygen of IMP, with subsequent cleavage of the carbon-oxygen bond. To postulate a phosphorylated enzyme intermediate, one would have to propose three phosphoryl transfers; however, it seems unlikely that there are two phosphoryl-enzyme intermediates.

Positional isotope exchange is a technique by which one can investigate the existence of phosphoryl intermediates in a reaction mechanism (6). By labelling the γ-phosphate of GTP with ^{18}O, one can observe the exchange of the label from the β-γ bridge position to the β nonbridge of GTP, if exchange does occur. If exchange does occur, it can be observed by two methods: mass spectroscopy and ^{31}P NMR spectroscopy. With the method of ^{31}P NMR spectroscopy, ^{18}O labelled in the β-γ bridge position can be differentiated from ^{18}O labelled in the β nonbridge position. The difference between these two resonances was found to be 0.012 ppm for ATP (7). If an exchange is observed, one can hypothesize a phosphoryl-enzyme or a phosphoryl-substrate as a reaction intermediate.

Positional isotope exchange experiments were performed with adenylosuccinate synthetase to determine the reaction mechanism. We report that an exchange reaction occurs when [γ-^{18}O]GTP is incubated with IMP. No exchange reaction was observed when IMP was absent. These findings support a reaction mechanism that involves a 6-phosphoryl-IMP intermediate.

EXPERIMENTAL PROCEDURES

Adenylosuccinate synthetase was purified from rat muscle by the method of Baugher (8) and was dialyzed against a buffer containing 100 mM Hepes (pH 7.0) and 5 mM DTT. $[^{18}O]H_2O$ (97 atom % purity) was obtained from MSD Isotopes (Merck and Co.). CDTA, DTT, GDP, GTP, Hepes were purchased from Sigma Chemical Co. Solvents used in the synthesis of $[\gamma\text{-}^{18}O]$GTP were dried, distilled, and stored in a desiccator with P_2O_5 before use. Other chemicals were of the highest grade commercially available and were used without further purification.

$[^{18}O]H_3PO_4$ was synthesized by the method given in Rhyu et al. (9) from PCl5 (which was previously purified by sublimation), and purified by the method in Hackney et al. (10). $[\gamma\text{-}^{18}O]$GTP was synthesized by the method of Wehrli et al. (11), except as noted below. GDP-morpholidate was eluted from a 2.5 x 15 cm column of AG 1X2 (HCO_3^- form) by a 4-liter linear gradient from 5 to 750 mM TEAB. The $[\gamma\text{-}^{18}O]$GTP was purified by elution from a 2 x 34 cm column of DEAE-cellulose (HCO_3^- form) by a 3-liter linear gradient from 5 to 360 mM TEAB. The labelled GTP was assayed spectrophotometrically and enzymatically to give a yield of 62 µmol. This represents a yield of 52% from GDP-morpholidate.

All the NMR experiments were performed on a Bruker 300 Fourier Transform NMR spectrometer operating at 121.5 MHz. Data were collected over 1600 scans, with a pulse width of 24 µsec (approximately a 60° pulse angle). The data were transformed by using a 0.2 Hz line broadening. All samples were incubated at 22° for 17 hours. (The enzyme showed no loss of activity when incubated at 22° for 60 hours.) The reactions were stopped

by adding CDTA (adjusted to pH 7.0) to give a final concentration of 30 mM. The total volume was adjusted to 2.0 ml. The samples then were stored on ice until ready for NMR analysis. The samples also carried a capillary containing 10% H_3PO_4 during data acquisition. The phosphoric acid resonance was used as an external standard and set to 0.0 ppm.

RESULTS

Fig. 1, A and B show the purity of the isotopic enrichment of the [γ-^{18}O]GTP alone (Fig. 1A) and with approximately equimolar unlabelled GTP added (Fig. 1B). The total shift between major peaks of the γ-^{31}P spectrum was 0.105 ppm. The value reported for ATP is 0.085 ppm (7). In the β region of the spectrum, the difference was 0.018 ppm, compared with a literature value of 0.016 ppm for ATP. The minor resonance seen in the γ spectrum was found to have a shift of 0.027 ppm. This compares quite well with the expected shift for a nonlabelled oxygen in the γ nonbridge position. Using the integrated areas of the peaks, we found the isotopic enrichment of the γ-phosphate to be 91.2%.

Fig. 2 depicts the spectra of the β peak of labelled GTP along (A), after incubation with adenylosuccinate synthetase (B), after incubation with 1.5 mM IMP (C), and after incubation with 1.5 mM IMP and 10 mM succinate (D). Fig. 3 illustrates the γ resonances for the same set of experiments. Succinate, a competitive inhibitor for aspartate (12), decreased the extent of exchange as compared with incubation with IMP alone. Table I summarizes the differences in the peak positions for all the experiments conducted. From these data, it can be seen that there is no exchange in the absence of IMP or in the presence of ligands that are known to bind to the enzyme in the absence of IMP (12). When IMP is present, there was an exchange of isotope between the β-γ bridge and the β nonbridge positions of GTP. This exchange was decreased when succinate was added to the incubation medium. In the experiment in which all the substrates were present (data not presented),

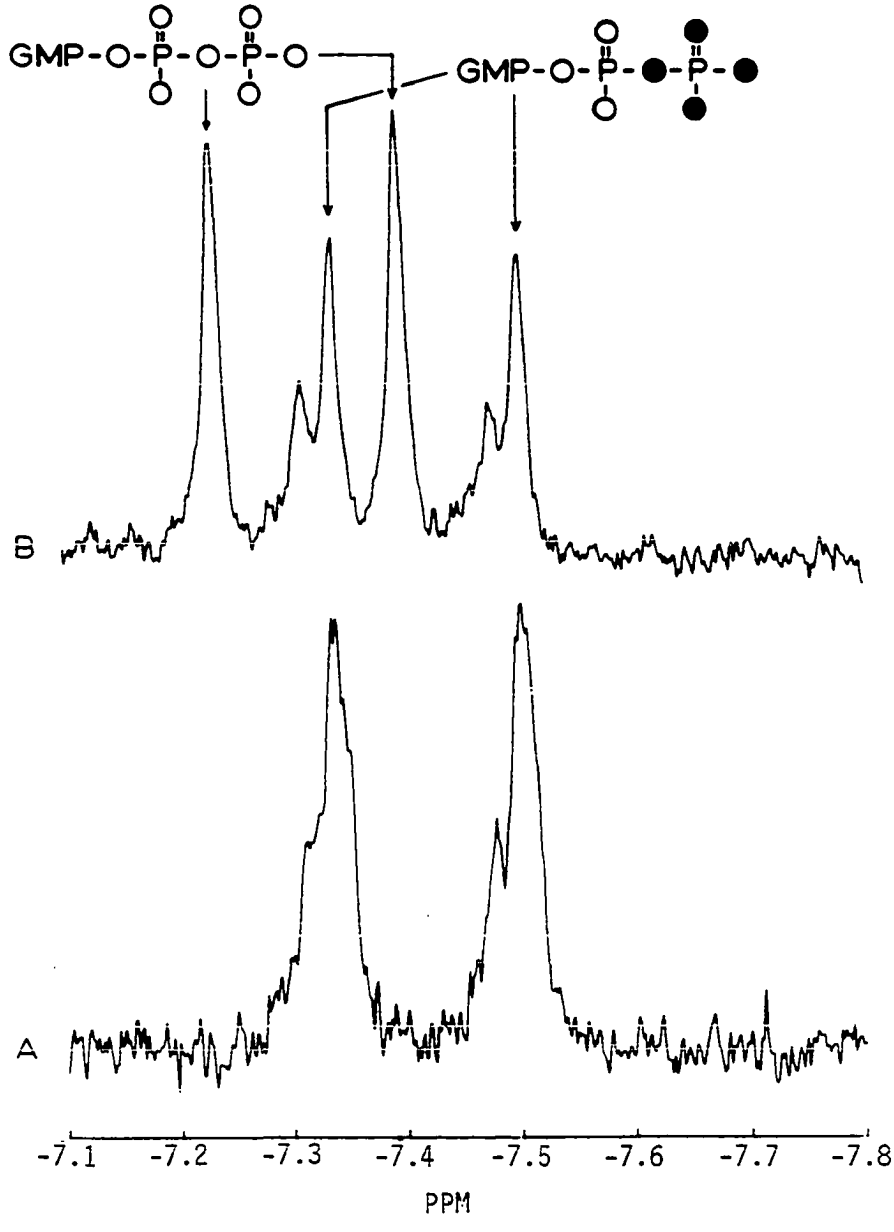


Fig. 1. ^{31}P NMR spectrum at 121.5 MHz of the γ-phosphorus group of 1.5 mM [γ-^{18}O]GTP (1A) and with 1.5 mM unlabelled GTP (1B), where ^{18}O is represented by the closed circles

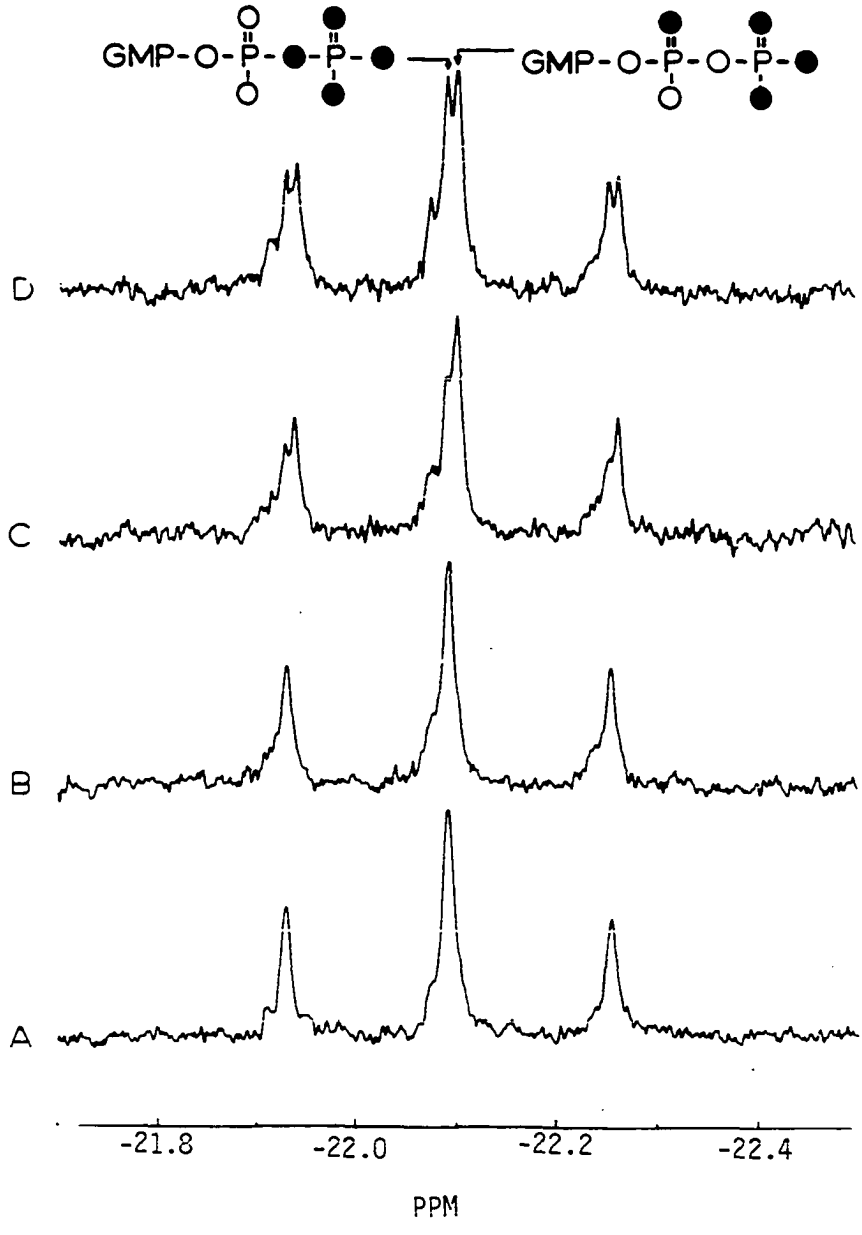


Fig. 2. ^{31}P NMR spectrum at 121.5 MHz of the β phosphorus group of 1.5 mM [γ-^{18}O]GTP (2A), after 17 hours of incubation with adenylosuccinate synthetase (2B), with 1.5 mM IMP added with the enzyme (2C), and with 1.5 mM IMP and 10 mM succinate added with the enzyme (2D)

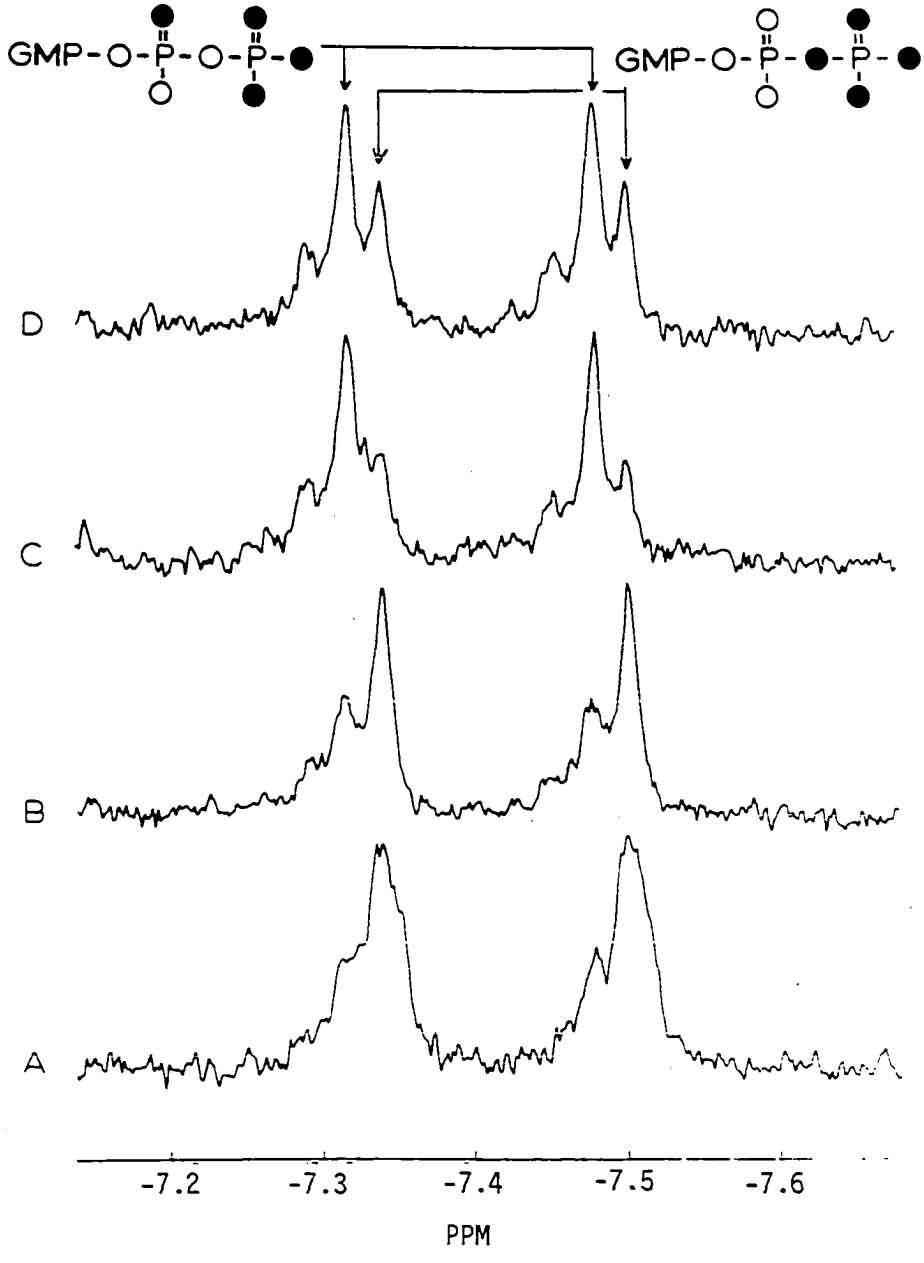


Fig. 3. ^{31}P NMR spectrum at 121.5 MHz of the γ-phosphorus group of 1.5 mM [γ-^{18}O]GTP (3A), after 17 hours of incubation with adenylosuccinate synthetase (3B), with 1.5 mM IMP added with the enzyme (3C), and with 1.5 mM IMP and 10 mM succinate added with the enzyme (3D)

Table I

Results of Positional Isotope Exchange

of [γ-^{18}O]GTP with Adenylosuccinate Synthetase

Additions[a]	Average Change in γ Peak Positions (ppm)	Average Change in β Peak Positions (ppm)
None	No exchange[b]	No exchange
1.5 mM IMP	0.020 ± 0.002	0.011 ± 0.002
2.0 mM aspartate	No exchange	No exchange
10 mM succinate	No exchange	No exchange
1.5 mM IMP + 10 mM succinate	0.020 ± 0.002	0.009 ± 0.002

[a]The reaction mixture contained 0.1 M Hepes (pH 7.0), 1.5 mM γ[^{18}O]GTP, 3.5 mM magnesium acetate, and 10% D_2O. The reactions were incubated with 0.16 units of adenylosuccinate synthetase for 17 hours at 22°. The reactions were stopped by the addition of CDTA to a final concentration of 30 mM. The total volume was adjusted to 2.0 ml.

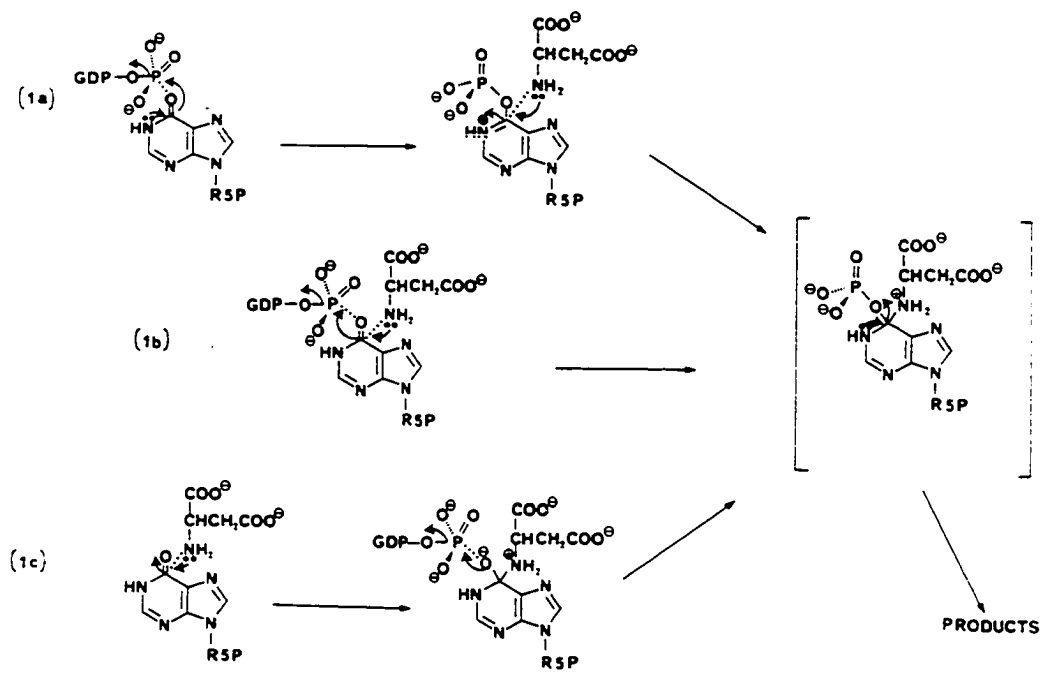
[b]No positional isotope exchange was observed under the assay conditions. No line broadening was used in these determinations.

an equilibrium mixture resulted. This led to the generation of peaks corresponding to phosphate with 0, 1, 2, and 3 ^{18}O atoms (13).

DISCUSSION

Three reaction mechanisms for adenylosuccinate synthetase have been suggested. In 1956, Lieberman proposed a 6-phosphoryl-IMP intermediate (2). This mechanism involves a nucleophilic attack by the 6-oxygen of IMP on the γ-phosphorus of GTP, generating the 6-phosphoryl-IMP intermediate. To date, this intermediate has eluded chemical synthesis. In the next step, the α-nitrogen of aspartate makes a nucleophilic attack on the 6-carbon of IMP, leading to a tetrahedral transition state. This breaks down to yield adenylosuccinate and orthophosphate. Lieberman based this mechanism on the finding that all the label in [6-^{18}O]-IMP ended up as [^{18}O]P_i and because there was no GTP $\rightleftharpoons$ [^{32}P]P_i exchange when aspartate was missing from the reaction mixture. Fromm (14) generated results from isotope exchange at equilibrium experiments that also support the hypothesis of a 6-phosphoryl-IMP intermediate. Lieberman's mechanism is depicted in 1a of Scheme 1.

In 1962, Miller and Buchanan suggested a concerted mechanism for adenylosuccinate synthetase (3). In this mechanism, the α-nitrogen of aspartate makes a nucleophilic attack on the 6-carbon of IMP at the same time that the 6-oxygen of IMP makes its attack on the γ-phosphorus of GTP, thus leading to the tetrahedral transition state. The mechanism was proposed to account for the finding that arsenolysis or phosphorolysis of adenylosuccinate does not occur when GDP is missing from the reaction mixture. It is also consistent with the lack of partial exchange reactions for adenylosuccinate synthetase. This mechanism is shown in part 1b of Scheme 1.



Scheme 1

A third mechanism was proposed by Markham and Reed in 1978. The first step of this mechanism has the α-nitrogen of aspartate attacking the 6-carbon of IMP creating an "oxy-anion" intermediate. The activated oxygen then makes a nucleophilic attack on the γ-phosphorus of GTP leading to the tetrahedral transition state. This study used GTPγS (guanosine-5'-O-(3-thiotriphosphate)), which lowers the turnover number of the enzyme by 12.5-fold. At this lower rate, Markham and Reed (4) were able to observe a transient in the UV spectrum. The attributed this transient to the oxy-anion intermediate, reasoning that phosphorylation of the N-6 of AMP leads to a spectral red shift that is in the opposite direction of the transient that was observed with GTPγS. The Markham and Reed mechanism is outlined in part 1c of Scheme 1.

The data from the experiments reported in this paper show that positional isotope exchange did take place between the β-γ bridge and the β nonbridge positions of GTP in the presence of adenylosuccinate synthetase. The finding that the exchange took place only in the presence of IMP is indicative of a 6-phosphoryl-IMP intermediate. This would support the mechanism originally proposed by Lieberman (2). The other two mechanisms can be eliminated since they require aspartate to be present before the GDPO-PO_3 bond is broken.

Data used to support other models can be interpreted to fit mechanism 1a of Scheme 1 as follows: The finding that arsenolysis does not occur without GDP being present (3) can be interpreted as a need by the enzyme for the guanosine nucleotide for catalytic activity. In the absence of GDP, the enzyme cannot enter into a catalytic conformation. This is

supported by the finding that GTP and IMP bind synergistically to the enzyme (4).

The sequential kinetics reported for adenylosuccinate synthetase from a variety of sources (8, 12, 15, 16, 17) are consistent with the participation of a 6-phosphoryl-IMP as an intermediate, if it is assumed that all substrates and products remain bound to the enzyme until adenylosuccinate is formed (18). This proposal also would account for the lack of partial exchange reactions.

The results of this report on positional isotope exchange, along with the findings of Webb et al. (5), which demonstrate the inversion of configuration when GTP is cleaved, are consistent with 6-phosphoryl-IMP being an intermediate in the adenylosuccinate synthetase reaction. Other proposals advanced to explain the mechanism of the reaction seem not in harmony with the results of the present investigation.

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SECTION II: TRANS-1,2-DIAMINOCYCLOHEXANE-N,N,N',N'-TETRAACETIC ACID IS SUPERIOR TO ETHYLENEDIAMINE-N,N,N',N'-TETRAACETIC ACID FOR SEQUESTERING Mg^{2+} IN ^{31}P NMR EXPERIMENTS INVOLVING ATP SPECTRA AT NEUTRAL AND ACIDIC pH

trans-1,2-Diaminocyclohexane-N,N,N',N'-Tetraacetic Acid Is Superior to Ethylenediamine-N,N,N',N'-Tetraacetic Acid For Sequestering Mg^{2+} in ^{31}P NMR Experiments Involving ATP Spectra at Neutral and Acidic pH

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ABSTRACT

The use of ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) to remove Mg^{2+} from samples containing ATP at acidic or neutral pH prior to ^{31}P NMR spectroscopic analysis leads to significant broadening of the gamma and beta phosphorus resonances of ATP as compared to ATP alone. We have found that the use of trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) reduces the broadening of the ATP resonances.

At pH 7.0, 30 mM EDTA in the presence of 5 mM ATP and 7 mM Mg^{2+} leads to a 3-fold increase in the peak width of the gamma phosphorus of ATP as compared with 5 mM ATP alone. When 30 mM CDTA is used in the place of EDTA, the peak width decreased to about 80% of the peak width of ATP alone. When the experiment is repeated at pH 8.5, both EDTA and CDTA lead to narrow peak widths with no significant difference between the two spectra. At pH 6.0, the use of EDTA leads to a spectrum that is very noisy with a 10-fold increase in the peak width as compared with ATP in the absence of Mg^{2+} at this pH.

These results do not reflect the differences in the chelating strength between EDTA and CDTA: The free Mg^{2+} concentration in the presence of each chelator, as calculated by the computer program given in the Appendix, was nearly equal at each pH. The results, however, reflect a difference in the lability of the metal-ligand bond between EDTA and CDTA.

INTRODUCTION

In conducting isotope scrambling experiments with adenylosuccinate synthetase (EC 6.3.4.4), we found the use of ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA) to be totally unsatisfactory for resolving the ^{31}P nuclear magnetic spectroscopy (NMR) resonances (1). We have, therefore, sought a chelating agent that is more effective than EDTA in a pH range at which most enzymes function. The most effective compound that we have found for this purpose is trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA).

It is well known that paramagnetic metal ions, such as Mn^{2+}, broaden spectral lines by shortening the spin-spin relaxation time of diamagnetic nuclei like ^{31}P in ATP. Other divalent metal ions, such as Mg^{2+}, can cause broadening of spectral lines through exchange broadening. Misawa et al., found that Mg^{2+} can broaden the beta and gamma resonances of ATP through a bimolecular exchange reaction: MgATP + ATP $\rightleftharpoons$ ATP + MgATP (2). This exchange leads to broadened peaks if the exchange is in the intermediate exchange region on the NMR timescale. Consequently, it is often necessary in NMR experiments to either purify the material before actual spectral determination or, alternatively, to add a chelating agent in order to obtain useful spectra. This problem is particularly acute when studying enzymes like phosphotransferases that require divalent metal ions for catalytic activity. To circumvent isolation, a procedure that may cause destruction of labile phosphate esters, EDTA is frequently added to the assay mixtures preceding NMR analysis. In other experiments where the presence of an enzyme is required during analysis, isolation of the

nucleotide cannot be performed (3). We have found that the use of EDTA at neutral or acidic pH leads to broadened spectral resonances. For experiments that require resolution of narrowly separated resonances, a better chelator is required. CDTA was found to meet these requirements and has been used in this laboratory to study isotope scrambling with adenylosuccinate synthetase (1).

EXPERIMENTAL PROCEDURES

ATP, CDTA, Hepes, Mes, Tris (as TRIZMA base) were purchased from Sigma Chemical Co. Gold Label magnesium acetate (99.999%) was obtained from Aldrich Chemical Co. Certified A.C.S. grade magnesium acetate and EDTA were obtained from Fisher Scientific. All other chemicals were of A.C.S. grade and used without further purification. All NMR experiments were conducted on a Bruker WM-300 Fourier Transform NMR Spectrometer operating at 121.5 MHz. Data were collected over 1000 scans with a pulse width of 17 μsec (a 45° pulse angle); 16 K data points were taken yielding a digital resolution of 0.0034 ppm. The data were transformed by using 0.2-Hz line broadening. All samples contained 10% D_2O, 5 mM ATP, 100 mM buffering salt, and when required, 7 mM magnesium acetate, 30 mM EDTA, and 30 mM CDTA. The buffering salts were Mes for pH 6.0, Hepes for pH 7.0, and Tris for pH 8.5. In the experiments using manganese (II) acetate, the samples contained 10% D_2O, 10 mM ATP, 100 mM Hepes (pH 7.0), and when required, 50 μM manganese (II) acetate, 30 mM EDTA, and 30 mM CDTA. EDTA and CDTA solutions were made separately for each pH and preequilibrated to the proper pH. The samples carried a capillary containing 10% H_3PO_4 during data acquisition, and it was used as an external standard and set to 0.0 ppm.

RESULTS AND DISCUSSION

In experiments such as positional isotope exchange, one must be able to resolve small differences in resonance frequency. For ^{18}O labelled ATP, this difference often can be as low as 0.012 ppm (4). To obtain this resolution, one must use a NMR spectrometer with a large magnetic field and remove divalent and paramagnetic ions from the assay solution as these ions are known to increase spectral line widths. To eliminate divalent metal ions, one commonly purifies the nucleotide from the reaction mixture or adds chelating agents, such as EDTA, to the solution before analyzing the sample. In this laboratory, we have found EDTA to be totally unsatisfactory for use in positional isotope exchange studies with [γ-^{18}O]GTP and adenylosuccinate synthetase. We found that CDTA was much more satisfactory for the resolution of the phosphorus resonances of GTP (1). This report quantitates the differences between these two chelators, EDTA and CDTA. In addition, these findings may be of more general interest to investigators who study phosphotransferases using NMR.

Fig. 1 shows a comparison of the γ-phosphorus resonances of ATP at pH 7.0 with EDTA and CDTA as chelating agents. Fig. 1A shows the spectrum of 5 mM ATP. Fig. 1B depicts the same resonance in the presence of 7 mM Mg^{2+}. As can be seen, the resonances are shifted downfield by 2.2 ppm. This is due to a deshielding effect on the phosphorus atom caused by Mg^{2+} chelating to the β and γ phosphates of ATP. This value agrees with the data presented by Cohn and Hughes (5) which used 20 times the concentration of ATP. The peaks were significantly broadened from 0.021 ppm for ATP alone to 0.072 ppm in the presence of Mg^{2+} (see Table I). The sensitivity of the

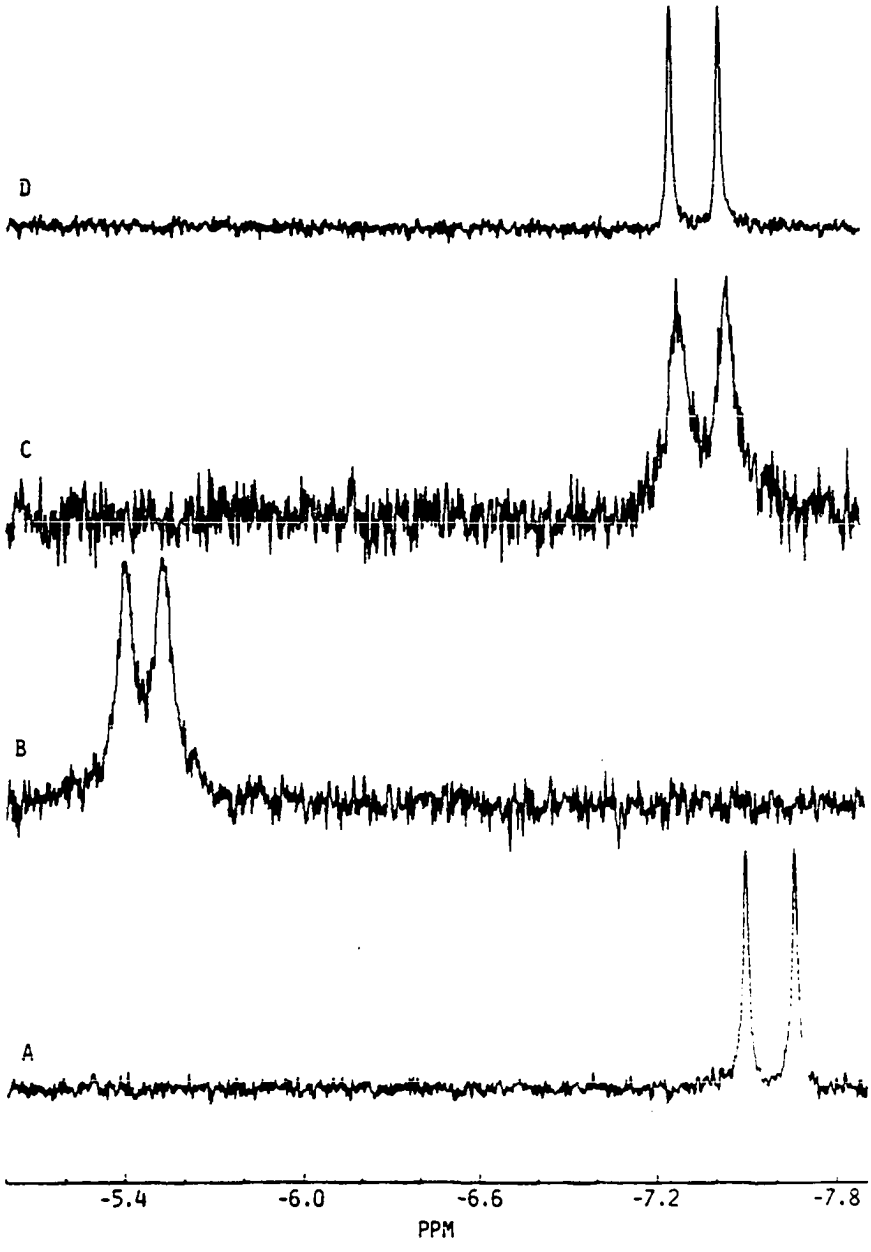

Fig. 1. ^{31}P NMR spectra at 121.5 MHz of the γ-phosphorus resonance of 5 mM ATP at pH 7.0 (A), plus 7 mM magnesium acetate (B), plus 30 mM EDTA (C), and plus 30 mM CDTA instead of 30 mM EDTA (D)

Table I

Effect of Metal Chelators on ^{31}P NMR Resolution

	pH 6.0		pH 7.0		pH 8.5	
Additions[a]	Sensitivity[b]	Peak Width[c]	Sensitivity	Peak Width	Sensitivity	Peak Width
none	24.5	0.040	34.2	0.021	31.3	0.021
Mg^{2+}	10.3	0.253	9.3	0.072	21.2	0.090
Mg^{2+} + EDTA	3.7	0.430	8.6	0.064	69.4	0.013
Mg^{2+} + CDTA	9.2	0.060	35.2	0.017	69.4	0.012

[a]Additions to 5 mM ATP, 10% D_2O, 100 mM buffering salt. The buffering salt was Mes at pH 6.0, Hepes at pH 7.0, and Tris at pH 8.5. The concentration of the other compounds were: Mg^{2+}, 7 mM; EDTA, 30 mM; and CDTA, 30 mM.

[b]Calculated using the equation for sensitivity given in reference 6.

[c]Peak widths were calculated for the γ-phosphorus resonance of ATP. The values are uncorrected for the 0.2-Hz line broadening used during spectral transformation. Peak width units are given in ppm.

measurement, an estimate of the signal-to-noise ratio, was calculated for the equation found in reference (6):

$$\text{Sensitivity} = \frac{(\text{peak height})\ (2.5)}{(\text{peak to peak noise level})}$$

The sensitivity was dramatically decreased from 34.2 to 9.3 in the presence of Mg^{2+}.

When 30 mM EDTA was added (Fig. 1C), the γ-phosphorus resonance shifted back upfield to near the resonance obtained with ATP alone. The peak widths decreased slightly from 0.072 ppm with Mg^{2+} to 0.064 ppm with EDTA. But the sensitivity also decreased slightly from 9.3 with Mg^{2+} to 8.6 with EDTA. Comparing the spectrum containing EDTA with the spectrum for ATP alone, one finds that the noise level has greatly increased (the sensitivity dropped from 34.2 to 8.6 with EDTA). The peak widths of the spectrum with EDTA are three times larger than the same peaks in the spectrum of ATP alone.

If 30 mM CDTA is added instead of EDTA (Fig. 1D), the peak widths narrow remarkably. Comparing the spectra of CDTA and EDTA, one notes that the peak widths decreased from 0.064 ppm to 0.017 ppm with CDTA. The sensitivity increased dramatically from 8.6 to 35.2 with CDTA. Comparing the spectrum taken in the presence of CDTA with the spectrum of ATP alone, one sees a slight increase in sensitivity (34.2 to 35.2 with CDTA). These is also a slight decrease in the peak width from 0.021 to 0.017 ppm with CDTA. This small broadening of the peak width of ATP alone may be due to traces of metals that have been reported to be present in commercial ATP (7), and are effectively chelated with CDTA. The same effects seen with

the γ-phosphorus resonance of ATP are also seen in the β resonance.

When either EDTA or CDTA was incubated with ATP alone (data not shown), the was a slight narrowing of the peaks and a downfield shift of the γ resonances of ATP. These results demonstrate that paramagnetic impurities were not introduced with the chelating agents. To further test for paramagnetic impurities, the experiments at pH 7.0 were repeated using 99.999% magnesium acetate instead of reagent grade magnesium acetate. These results showed no significant differences in the spectra recorded with either of the magnesium salts.

The differences between the spectra with EDTA and with CDTA are not just due to the differences in the chelating strength of the two ligands. Table II shoes the apparent association constants and free Mg^{2+} concentrations calculated to be present at each pH used in this study. (The computer program used to calculate these values can be found in the Appendix.) As can be seen from this table, the concentration of free Mg^{2+} is nearly the same in the presence of either of the two chelators. The broadening of the γ-phosphorus resonance in the presence of EDTA must be due to more than just the free Mg^{2+} calculated to be present. Otherwise, the spectrum with CDTA would have been broadened more than the spectrum of ATP alone.

Day and Reilley have reported that he metal-ligand bond of EDTA is much more labile than the metal-ligand bond of CDTA (8). By studying the interconversion rates between the acetate carbons, Day and Reilley found that EDTA interconverts much more rapidly than CDTA. This was attributed to two causes: First, the cyclohexane ring of CDTA provides steric

hindrance to a water molecule attempting to displace the coordinated nitrogen atom. Second, the cyclohexane ring is a much more rigid structure than the ethylenic backbone of EDTA. The rigid structure of CDTA lowers the rotational freedom of the coordinated atoms. This restricts the acetate groups from rapidly interconverting when the metal-nitrogen bond is broken (8).

In experiments reported here, the exchange rate of free Mg^{2+} with chelated Mg^{2+} can be indirectly measured by its interaction with the phosphate groups of ATP. Mg^{2+} exchanging with ATP and MgATP exchanging with ATP leads to the broadening of the resonances seen in Fig. 1B. But if the Mg^{2+} concentration is held low enough, the amount of MgATP seen in the NMR will be very low. This will lead to narrower peaks in the NMR spectrum (Fig. 1A). If Mg^{2+} is chelated to a compound that has a very low exchange rate, the amount of Mg^{2+} able to exchange broaden the ATP resonances will be very low. This will also lead to narrow peaks in the NMR spectrum (e.g., Fig. 1D). If on the other hand, the chelator has a high exchange rate, then the number of Mg^{2+} atoms able to exchange with ATP and broaden the resonances will increase even though the concentration of free Mg^{2+} is the same as the low-exchange-rate chelator. This evidently is what happened when EDTA is used as a chelator (Fig. 1C); i.e., the peaks are broadened. When CDTA is used, the exchange rate is much slower, and so, the peaks are narrower than with EDTA.

It has also been shown that with Fe^{3+}, a ternary complex can form with ATP and EDTA (9). Elgarvish and Granot demonstrated that this complex was monodentate with respect to ATP with the Fe^{3+} binding at the γ phosphate of

Table II

Apparent Stability Constants and Free Mg^{2+} Concentrations for MgEDTA and MgCDTA at pH 6.0, 7.0, and 8.5

		pH 6.0		pH 7.0		pH 8.5	
Ligand	log K_{True}[a]	log K_{App}[b]	Free Mg[c]	log K_{App}	Free Mg	log K_{App}	Free Mg
EDTA	8.79	4.16	18.5	5.49	0.948	7.04	0.028
CDTA	11.02	4.24	15.8	5.56	0.808	7.12	0.023

[a]True stability constants obtained from reference 10.

[b]Apparent stability constant calculated by the computer program given in the Appendix using the equation given in reference 11. Value expressed as the log of molar units.

[c]Concentration calculated considering 30 mM ligand, 5 mM ATP, and 7 mM Mg^{2+} using the program given in the Appendix. The apparent stability constant for ATP was calculated using the equation and values given in reference 12. The concentration is expressed in μM units.

ATP. To see if this was happening with Mg^{2+}, the β-phosphorus resonance was examined. The peak width of the β phosphorus increased from 0.016 ppm for ATP alone to 0.087 ppm in the presence of Mg^{2+} and EDTA. This demonstrates that a ternary complex like the one that forms with Fe^{3+} does not form with Mg^{2+}.

When the experiment was repeated at pH 8.5, the resolution was significantly increased when the chelators were present (see Table I). When 7 mM Mg^{2+} was added to the ATP solution, the peak widths dramatically increased from 0.021 ppm to 0.090 ppm. The sensitivity decreased from 31.1 to 21.2 with Mg^{2+}. When EDTA was added to the ATP and Mg^{2+} solution, the resolution increased. The peak widths decreased from 0.090 ppm with Mg^{2+} to 0.013 ppm when EDTA was added. Comparing the spectrum with ATP alone with that obtained after EDTA was added, one observes a sharper spectrum. The peak widths decreased from 0.021 ppm to 0.013 ppm. The sensitivity increased 2.2 times from that with ATP alone. The spectrum taken when CDTA was used in place of EDTA yielded a spectrum virtually identical to the spectrum obtained with EDTA.

When the experiment was conducted at pH 6.0, neither EDTA nor CDTA gave satisfactory results (Fig. 2). The γ phosphorus of ATP (Fig. 2A) gave a peak width of 0.040 ppm and a sensitivity of 24.5. When 7 mM Mg^{2+} was added, the peaks were so broadened that only one large resonance was observed (Fig. 2B). The peak width increased to 0.253 ppm in the presence of Mg^{2+} while the sensitivity dropped to 10.3. As at pH 7.0 and 8.5, there was a downfield shift of the resonances in the presence of Mg^{2+}. When 30 mM EDTA was added to the assay solution (Fig. 2C), the peak was so

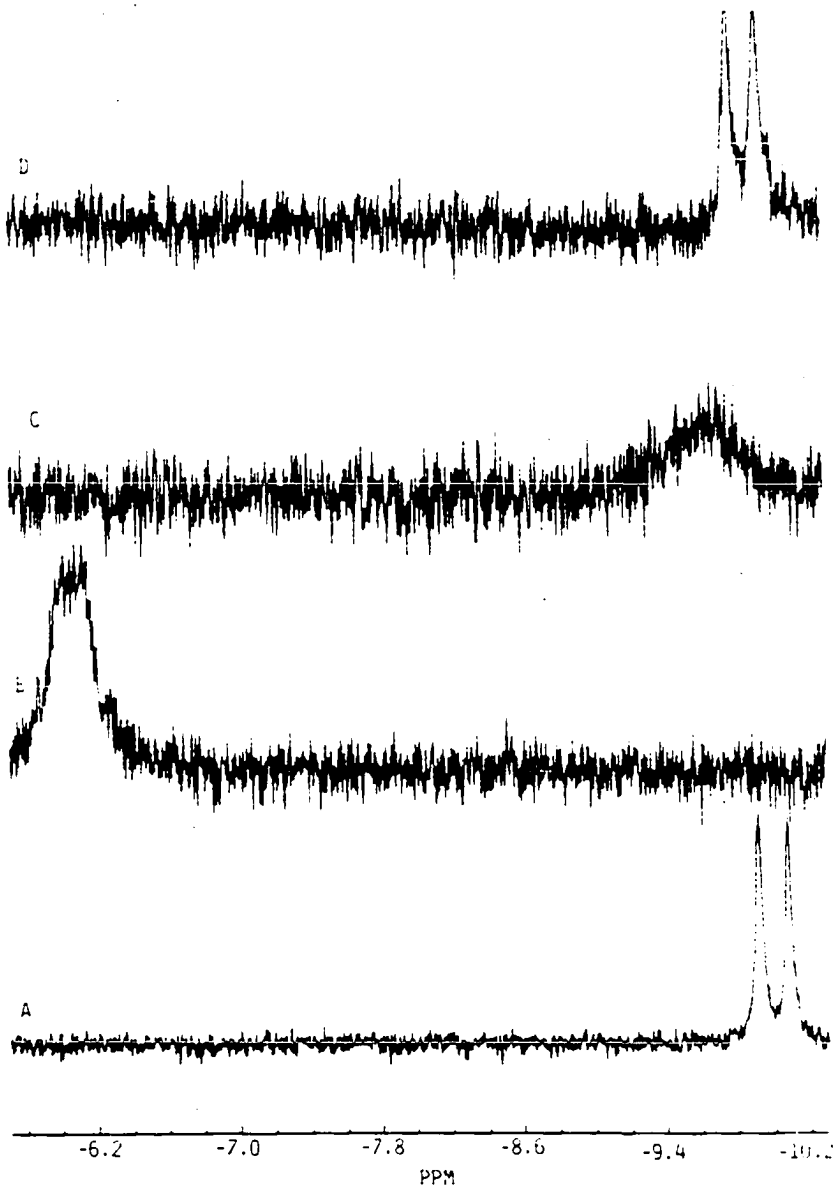

Fig. 2. ^{31}P NMR spectra at 121.5 MHz of the γ-phosphorus resonance of 5 mM ATP at pH 6.0 (A), plus 7 mM magnesium acetate (B), plus 30 mM EDTA (C), and plus 30 mM CDTA instead of 30 mM EDTA (D)

broadened as to be almost unresolved. The peak width increased to 0.430 ppm. The sensitivity dropped from 10.3 with Mg^{2+} to 3.7 with EDTA. Although the resonance was shifted back upfield from that obtained in the presence of Mg^{2+}, the signal was so broadened that the spectrum was extremely difficult to evaluate. When 30 mM CDTA was added in place of EDTA (Fig. 2D), the resolution increased dramatically, and the two γ resonances were resolved. The peak width decreased to 0.060 ppm while the sensitivity increased to 9.2 as compared with EDTA. If one compares the spectrum obtained with CDTA with that of ATP alone, the spectrum with CDTA was broader (1.5 times broader) and noisier (the sensitivity decreased 2.7 fold). This effect may be due to the 17 µM free Mg^{2+} calculated to be present at this pH.

The experiments at pH 7.0 were repeated to determine the effect paramagnetic ions have on the phosphorus resonances of ATP. Mn^{2+} (50 µM) was used in place of the Mg^{2+} used previously. Fig. 3A shows the spectrum of 10 mM ATP. Fig. 3B illustrates the spectrum when 50 µM Mn^{2+} was added. It can be seen that the α, β, and γ peaks were significantly broadened. This is in contrast to the case with Mg^{2+} where the width of the α peak remained constant throughout the experiments. When 30 mM EDTA was added (Fig. 3C), the peaks sharpened back to the original shape. The peak width of the γ-phosphorus resonance did broaden slightly as compared to ATP alone (from 0.017 ppm to 0.022 ppm in the presence of EDTA). When 30 mM CDTA was used instead of EDTA (Fig. 3D), the spectrum was essentially the same as with EDTA. These experiments show that EDTA and CDTA are both able to reduce the broadening effect caused by Mn^{2+}. These findings also serve to

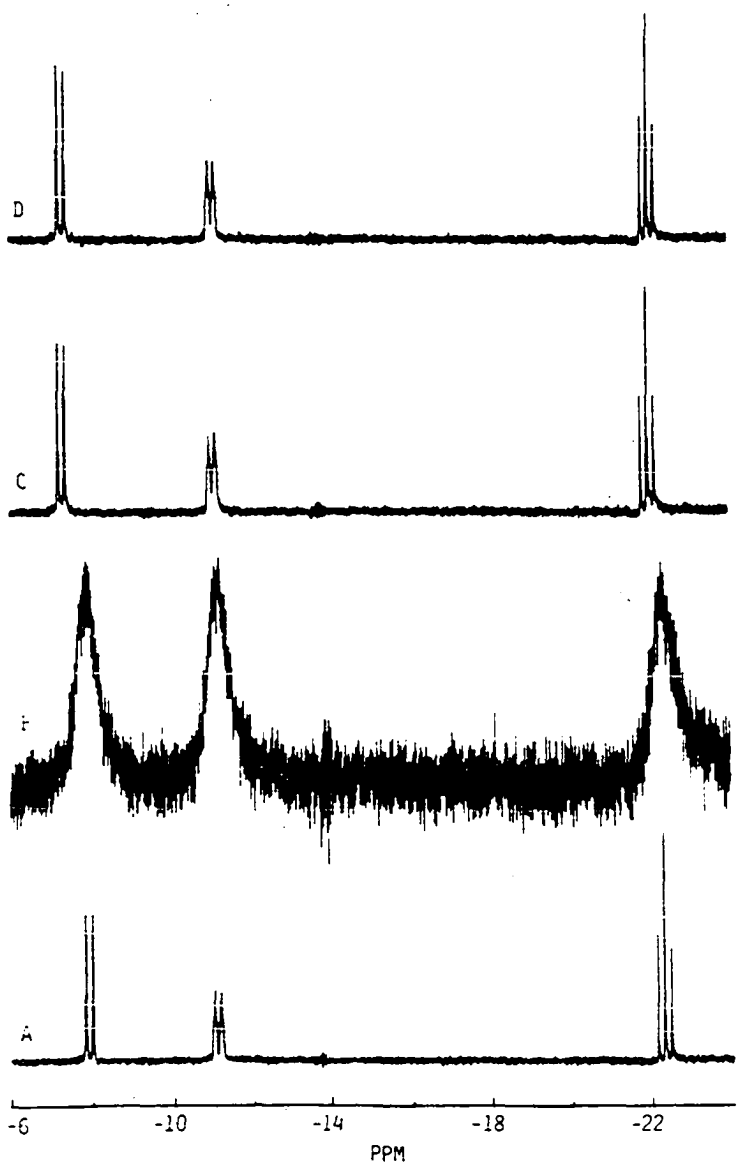

Fig. 3. ^{31}P NMR spectra at 121.5 MHz of the phosphorus resonances of 10 mM ATP at pH 7.0 (A), plus 7 mM manganese acetate (B), plus 30 mM EDTA (C), and plus 30 mM CDTA instead of 30 mM EDTA (D)

eliminate the possibility that the results obtained with Mg^{2+} are caused by paramagnetic impurities.

When one compares the experiments with EDTA and CDTA at pH 6.0, 7.0, and 8.5, a trend is clearly observable. With EDTA at pH 8.5, the signal is very sharp. As the pH is lowered, the signal becomes more and more noisy. This is due, as stated earlier, to the exchange of Mg^{2+} from the chelated structure, MgEDTA, into solution. From these experiments, MgEDTA seems to be much more labile at acidic pH than at basic pH. Comparing CDTA over the same range, one does not see the same exchange broadening. The broadening of the γ resonances seen at pH 6.0 may be due to the lower chelating strength of CDTA at this pH.

For experiments requiring high resolution of peak resonances, these findings show that CDTA is much superior to equimolar EDTA at neutral pH. If experimental conditions allow one to work at higher pH, then the choice between EDTA and CDTA is not as important. When working at a pH more acidic that 8.5, the differences between these chelators becomes more dramatic. Consequently, great care in the choice and concentration of a chelator is very important.

When studying enzyme reactions where the pH optimum is near 7, the choice between chelators is straightforward. CDTA leads to a much sharper spectrum than EDTA under the same conditions (Figs. 1C, D). This finding is especially important because EDTA is frequently used to stop enzymatic reactions involving nucleoside triphosphates. In this laboratory, CDTA was found to significantly improve the resolution of the phosphorus resonances of GTP (unpublished results) as well as ATP.

These results can be extended to studying the interconversion rates between enzyme-bound substrates and products. Measuring the interconversion rate is dependent on being able to accurately measure the peak width of the substrates during exchange and when the compounds are bound but exchange-inactive. With phosphotransferases, this is easily done by removing the divalent metal ion. These results suggest that CDTA would be much more effective than EDTA near pH 7.

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APPENDIX

```
PROGRAM EQUILIBRIUM;
(* This program is designed to return values for the apparent equilibrium*
 * constants for metal chelators.                                        *
 * Values needed to run this program are:                                *
 *  the first three proton association constants (in log form)           *
 *  the true metal-ligand association constant (in log form)             *
 *  the pH at which the data is to be calculated                         *
 *  the concentration of ligand                                          *
 *  the concentration of metal ion                                       *
 *                                                                       *
 * The varibles used in this program are:                                *
 *   K1C,K2C,K3C  Logs of the proton-CDTA association constants          *
 *   K1E,K2E,K3E  Logs of the proton-EDTA association constants          *
 *   KTrueCDTA    Log of the true Metal-CDTA association constant         *
 *   KAppCDTA     Log of the apparent M-CDTA association constant         *
 *   KTrueEDTA    Log of the true Metal-EDTA association constant         *
 *   KAppEDTA     Log of the apparent Metal-EDTA association constant     *
 *   KCorrATP     Log of the MgATP assoc. constant corrected to pH 8.0    *
 *   KAppATP      Log of the apparent metal-ATP association constant      *
 *   pH           pH of the solution                                      *
 *   HConc        Proton concentration of the solution                    *
 *   LigandConc   Concentration of the Ligand                             *
 *   MetalConc    Metal ion concentration                                 *
 *   FreeMetal    Free Metal ion concentration                            *
 *   ATPConc      ATP concentration                                       *
 *                                                                       *
 * All concentrations are expressed in mM.                               *
 * The association constants are expressed in molar units.               *
 * The constants declared in the CONST block are correct when the metal  *
 *    ion is Magnesium.                                                  *)


USES              (* This block is ONLY used in Apple Pascal *)
   TRANSCEND;

CONST
   LigandConc = 0.030;
   MetalConc = 0.007;
   ATPConc = 0.005;
   KTrueCDTA = 11.02;
   KTrueEDTA = 8.79;
   K1C = 12.4;
   K2C = 6.15;
   K3C = 3.53;
   K1E = 10.24;
   K2E = 6.16;
```

```
   K3E = 2.66;
   KCorrATP = 4.86;

VAR
   MLComplex,MAComplex,KAppCDTA,KAppEDTA,KAppATP,FreeMetal,pH,HConc : REAL;
   Ans : CHAR;

FUNCTION ALOG(Dummy:REAL):REAL;
   (* This function is an exponential function (base 10) which will *
    * return a value equal to 10 raised to the power of "Dummy".    *)

   CONST
      LogOfE = 0.4342945;

   BEGIN
      ALOG := EXP(Dummy / LogOfE);
   END; (* of ALOG *)

PROCEDURE FINDANSWER(K1,K2:REAL;VAR Free,LComplex,AComplex:REAL);
   (* This procedure evaluates the Free Metal concentration via an *
    * iterative procedure when given the apparent association      *
    * constants for the ligand and ATP, respectively.  Varibles    *
    * used in this procedure are:                                  *
    *   K1        Apparent assoc. constant for Ligand              *
    *   K2        Apparent assoc. constant for ATP                 *
    *   Free,M    Free metal concentration (M is calculation form) *
    *   LComplex  Metal-Ligand complex concentration               *
    *   AComplex  Metal-ATP complex concentration                  *
    *   Inc       Increment for finding M                          *
    *   P,Q       Intermediate values of EQ used to find the zero  *)

   CONST
      Tolerance = 1E-8;

   VAR
      C0,M,Inc,P,Q : REAL;

   FUNCTION SGN(X:REAL):INTEGER;
      (* This function returns a value of 1 if X is non-negative *)
      BEGIN
         IF X >= 0 THEN SGN := 1
         ELSE SGN := -1;
      END; (* OF SGN *)

   FUNCTION EQ(M:REAL):REAL;
      (* This is the equation for determining the free metal *
       * concentration in the presence of a chelator and ATP *)
      BEGIN
         EQ := MetalConc + (C0*(K1+K2) + K2*LigandConc + K1*ATPConc -1)*M
               + (K1*K2*C0 - (K1+K2))*M*M - K1*K2*M*M*M;
```

```
      END; (* OF EQ *)

   BEGIN (* of FINDANSWER *)
      (* Set starting parameters *)
      C0 := MetalConc - (LigandConc + ATPConc);
      K1 := ALOG(K1);
      K2 := ALOG(K2);
      M := 1E-6;
      Inc := (-1)*M/10;
      Q := EQ(M);
      REPEAT
         (* This is the iterative procedure which narrows *
          * in on the zero of EQ by varying M             *)
         P := Q;
         M := M+Inc;
         Q := EQ(M);
         IF SGN(Q) <> SGN(P) THEN
            Inc := (-1)*(Inc/10)
         ELSE IF ABS(Q) > ABS(P) THEN
            Inc := (-1)*Inc;
      UNTIL ABS(Q) < Tolerance;
      Free := M*1E+6; (* in microM units *)
      LComplex := (K1*M*LigandConc/(1+K1*M))*1000; (* in mM units *)
      AComplex := (K2*M*ATPConc/(1+K2*M))*1000;
   END; (* of FINDANSWER *)

FUNCTION APPARENT(True,K1,K2,K3:REAL):REAL;
   (* This function evaluates the apparent association constant *
    * given the true association constant and the proton        *
    * association constants.  Equation from JBC 255, 4087-4093  *)
   BEGIN
      APPARENT := True - LOG(1 + HConc*ALOG(K1) + HConc*HConc*ALOG(K1)
                  *ALOG(K2) + HConc*HConc*HConc*ALOG(K1)*ALOG(K2)*ALOG(K3));
   END; (* OF APPARENT *)

BEGIN (* of the main program *)
   REPEAT
      WRITE ('What is the pH of the solution? ');
      READLN (pH);
      HConc := ALOG(pH * (-1));
      KAppCDTA := APPARENT(KTrueCDTA,K1C,K2C,K3C);
      KAppEDTA := APPARENT(KTrueEDTA,K1E,K2E,K3E);
      KAppATP := KCorrATP - LOG((1+ALOG(6.95 - pH))/(1+ALOG(-1.05)));
      WRITELN ('At pH ',pH:4:2,' the KApp for MgATP is ',KAppATP:6:3);
      WRITELN;
      FINDANSWER(KAppCDTA,KAppATP,FreeMetal,MLComplex,MAComplex);
      WRITELN ('For CDTA, the parameters are:');
      WRITELN ('KApp (log of Molar units) = ',KAppCDTA:6:3);
      WRITELN ('Free Metal (microM units) = ',FreeMetal:7:4);
      WRITELN ('M-L Conc. (mM) = ',MLComplex:6:3);
```

```
      WRITELN ('M-ATP Conc. (mM) =',MAComplex:6:3);
      WRITELN;
      FINDANSWER(KAppEDTA,KAppATP,FreeMetal,MLComplex,MAComplex);
      WRITELN ('For EDTA, the parameters are:');
      WRITELN ('KApp = ',KAppEDTA:6:3);
      WRITELN ('Free Metal = ',FreeMetal:7:4);
      WRITELN ('M-L Conc.  = ',MLComplex:6:3);
      WRITELN ('M-A Conc.  = ',MAComplex:6:3);
      WRITELN;
      WRITE ('Do you wish another pH? (Y/N) ');
      READLN (Ans);
   UNTIL Ans = 'N';
   WRITELN ('Execution Completed.');
END.
```

SECTION III: OVERPRODUCTION, PURIFICATION, AND CHARACTERIZATION OF ADENYLOSUCCINATE SYNTHETASE FROM *ESCHERICHIA COLI*

Overproduction, Purification, and Characterization of Adenylosuccinate Synthetase from *Escherichia coli*

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ABSTRACT

Adenylosuccinate synthetase, encoded by the purA gene of Escherichia coli, catalyzes the first committed step toward AMP in the de novo purine biosynthetic pathway and plays an important role in the interconversion of purines. A 3.2-kilobase DNA fragment, which carries the purA gene, was cloned into the temperature-inducible, high-copy-number plasmid vector, pMOB45. Upon temperature induction, cells containing this plasmid produce adenylosuccinate synthetase at approximately 40 times the wild-type level. A scheme is presented for the purification of the overproduced adenylosuccinate synthetase to homogeneity in amounts sufficient for studies of its structure and mechanism. The wild-type and the overproduced adenylosuccinate synthetase enzyme preparations were judged to be identical by the following criteria. (1) The amino acid sequence at the N-terminus of the overproduced enzyme proved identical to the corresponding sequence of the wild-type enzyme. (2) Michaelis constants for both the wild-type and overproduced enzyme preparations were the same. And (3) both proteins shared similar chromatographic behavior and the same mobility during SDS polyacrylamide gel electrophoresis. Results from size-exclusion chromatography and SDS-polyacrylamide gel electrophoresis suggest that adenylosuccinate synthetase exists as a dimer of identical, 48,000-dalton, subunits.

INTRODUCTION

Adenylosuccinate synthetase [IMP: L-aspartate ligase (GDP forming), (EC 6.3.4.4)] catalyzes the first step in the synthesis of AMP from IMP. The reaction,

$$GTP + IMP + L\text{-}Aspartate \rightleftharpoons GDP + Adenylosuccinate + P_i$$

represents the first committed step towards AMP biosynthesis. Regulation of the enzyme reflects its central position in both de novo purine biosynthesis and the pathways of purine nucleotide salvage and interconversion (1). In Escherichia coli, feedback inhibition, substrate availability, and a specific inhibitor, guanosine 5'-diphosphate-3'-diphosphate (the stringent factor) can all function to control the enzyme's activity (1). In addition, the cell regulates the adenylosuccinate synthetase concentration at the level of gene transcription. At least five genetic loci, which specify enzymes of the de novo IMP biosynthesis, are regulated as a unit by ATP and a pur gene-specific repressor protein (2-6). The purA gene (adenylosuccinate synthetase) is also strongly repressed by excess adenine in the medium; however, derepression is observed at either low adenine or low guanine levels (7). The putative pur gene-specific repressor protein exhibits an ATP-dependent, but not GTP-dependent, binding to the purA gene (8); thus other regulatory proteins may also be involved.

The adenylosuccinate synthetase reaction also poses an interesting and general mechanistic problem. Steady-state kinetic analysis has

demonstrated that the enzyme has a sequential mechanism characterized by a completely random binding order for substrates and products (9). At least three mechanisms have been proposed: (a) a one-step "concerted" reaction (10), (b) a two-step mechanism with one intermediate, 6-phosphoryl-IMP (11, 12), and (c) a three-step reaction pathway in which an addition product between IMP and L-aspartate is initially formed, followed by its phosphorylation and decomposition into products (13). Studies from Reed's laboratory show that the adenylosuccinate synthetase reaction, using the chiral probe (γ-S)[$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O]GTPγS, proceeds with a net inversion of configuration at the γ-phosphorus (14). We recently showed, using the technique of positional isotope exchange, that 6-phosphoryl-IMP is a likely intermediate in the adenylosuccinate synthetase reaction (15). Additional assessment of the mechanism will require direct physical analysis of quaternary enzyme-substrate or enzyme-substrate-inhibitor complexes by such techniques as nuclear magnetic resonance spectroscopy and X-ray crystallography.

A major obstacle to investigations of adenylosuccinate synthetase has been the difficulty in obtaining large quantities of the purified enzyme. Studies of the enzyme from other sources indicate that it is present in cells in extremely low concentrations (1). Although adenylosuccinate synthetase from mammalian sources is easier to isolate, its usefulness for physical studies is limited by low solubility, i.e., 5 mg/ml. Here, we report the overproduction of E. coli adenylosuccinate synthetase, a simplified purification scheme, and a preliminary characterization of the enzyme.

EXPERIMENTAL PROCEDURES

Materials Chloramphenicol, DNase I, GTP, Hepes, IMP, IPTG, Phenyl-Sepharose CL-4B, and Tris (as TRIZMA base) were supplied from Sigma Chemical Company. The DNase I stock solution was made up to be 10 mg/ml and dialyzed into 100 mM NaCl, 50 mM Tris, 5 mM $MgCl_2$, pH 7.5. Dimethyl suberimidate was obtained from Pierce Chemical Company. All other chemicals were of the highest grade commercially available and used without further purification.

All HPLC runs were performed on a Waters HPLC system equipped with a 2-ml injection loop and was performed at room temperature. Flow rate was 1 ml/min. The HPLC solvents were filtered (0.45-μm Rainin Nylon 66 filters) and degassed before use. Samples were filtered through 0.22-μm filters (Millipore Millex-GV filters). Fractions were collected on ice.

Bacterial strains The following *E. coli* strains were utilized. H822: *thr*1, *leu*B6, *thi*1, *arg*43, *pro*A45, *pur*A46, *str*A126(*yps*L126), *ton*A25, *Tsx*71, T_2^R, T_3^R, *sup*E44(?) (Coli Genetic Stock Center, Strain Number 4436); H1238: *arg*I61, *arg*F58, *thr*25, *pur*A54, *ton*A49 (Coli Genetic Stock Center, Strain Number 5408) (16); and JM101: Δ*lac*-*pro*, *thi*, *sup*E, F'*tra*D36, *pro*AB, *lacI*^{q} ZΔM15 (17).

Growth of bacteria All bacterial strains were grown at the indicated temperature in LB medium (18) with the following exceptions: JM101 was stored on glucose-minimal media. H882 and H1238 were grown on

glucose minimal media plus adenine and appropriate amino acid supplements at the concentrations recommended by Davis et al. (19). Complementation of the purA mutations in H882 and H1238 was carried out by transforming pJS76 and scoring growth plus and minus adenine. Growth media were supplemented with antibiotics as follows: cells harboring pUC18, pUC19 -- 75 µg/ml ampicillin; and pMOB45 -- 7 µg/ml chloramphenicol or 15 µg/ml tetracycline. The induction of transcription from the lacZ promotor, in JM101(pJS76-18) and JM101(pJS76-19), was carried out by the addition of the lac inducer, IPTG, to 2 mM in the medium.

Preparation of crude enzyme fractions for adenylosuccinate synthetase assays At each time point, 20 ml of the culture was removed and put on ice. All subsequent steps were performed at 4°. The cells were centrifuged at 7000 x g for 10 min, resuspended in 1.0 ml 10 mM TE, and centrifuged for 5 min. in a microfuge. Next, the cells were resuspended in 0.4 ml 10 mM TE, and 50 µl of 1 mg/ml freshly prepared lysozyme (in 10 mM TE) was added and incubated for 20 min. The lysed cells were centrifuged in a microfuge for 15 min. The pellet was removed with a toothpick, and 0.75 ml saturated ammonium sulfate was added. The protein was centrifuged in a microfuge for 15 min. The pellet was resuspended in 80 mM KPi buffer (pH 7.0) and dialyzed against 1 l of 80 mM KPi buffer.

The standard assay for adenylosuccinate synthetase activity is the same as that of Rudolph (20), except that streptomycin sulfate was omitted. Assays performed on the crude enzyme (before the HPLC step) were corrected for the presence of aspartase. Aspartase activity was measured as

adenylosuccinate synthetase activity except the nucleotides were omitted from the assay solution. Activity is expressed in Units (μmoles adenylosuccinate formed per minute).

Plasmid DNAs pJS76-19, which is composed of a 3.2-kilobase, SspI-KpnI restriction fragment encoding the E. coli purA gene inserted into pUC19, was a gift from Dr. John M. Smith of Louisiana State University. The temperature-inducible, high-copy-number plasmid, pMO845 (21), was a gift from Dr. Arthur Kornberg, Stanford University. The pUC18 and pUC19 are standard DNA cloning vectors (22).

DNA cloning methods The following standard procedures were carried out according to Maniatis et al. (23): agarose gel electrophoresis, gel-isolation of DNA restriction fragments, DNA ligations, DNA-mediated transformations of E. coli, and DNA purification.

Vector construction We have found that the plasmid, pJS76-19 (Fig. 1), transformed the E. coli strains H882 (purA46) and H1238 (purA54) to adenine prototrophy at a high frequency and thus was judged likely to encode adenylosuccinate synthetase. Construction of the overproducing vector was as follows: pJS76-19 DNA was linearized with BamHI and then partially digested with HindIII under conditions that maximized the yield of the 3.2-kilobase DNA fragment. This purA-encoding DNA insert was purified from an agarose gel and ligated to HindIII / BamHI double-cut pMO845 DNA to generate pMS204. In a separate experiment, the 3.2-kilobase

DNA insert was ligated to HindIII / BamHI double-cut pUC18 DNA to generate pJS76-18. (See figure 1 for restriction maps.) The three constructions, pMS204, pJS76-18, and pJS76-19, were transformed into JM101 for assessment of enzyme overproduction.

Purification of adenylosuccinate synthetase Preparative growth of the JM101 (pMOB45-purA) strain was carried out as follows. The culture medium contained 500 ml of LB broth plus 12.75 g/l chloramphenicol (in a 2-liter flask) and was inoculated with 0.4 ml of an overnight culture. The culture was grown at 28° with vigorous shaking until the culture reached 0.1 OD_{595} (usually about 3.5 hr). Then, the culture was shifted to 42° and grown for 1.5 hr. At this time, 10 ml of 25% glucose was added, and the culture grown for an additional 1.5 hr. When the culture had reached the end of log phase, it was placed on ice. The cells were pelleted by centrifugation at 9000 x g for 10 min. The cells from 4 liters of culture were resuspended in 200 ml of 50 mM KPi and 10 mM EDTA (pH 7.0). The resuspended cells could be stored by freezing at -20°. If this was done, 25% glycerol was added to the resuspension buffer.

Adenylosuccinate synthetase was purified by a modification of the method of Rudolph (20). The cells from 4 liters of culture were thawed and centrifuged at 7000 x g for 10 min. The cells were resuspended in 120 ml 5 mM KPi buffer and placed in 6 centrifuge tubes (20 ml each). The cells were lysed by adding to each tube 0.5 ml of 4 mg/ml lysozyme freshly prepared in 5 mM KPi buffer. With the lysozyme, 8 µl of 0.25 M PMSF (in 95% ethanol) was added to inhibit proteinases. The lysate was incubated at

room temperature for 20 min with occasional inversion. Then, the cells were heated in a 50° water bath for 3 min to complete the lysis. To each tube, 100 µl of diluted DNase I (stock DNase I was diluted by adding 50 µl to 650 µl of 1.0 M $MgCl_2$) was added and incubated for 10 min at room temperature. The DNase digested the DNA allowing greater recovery of the initial supernatant fluid which would otherwise be trapped in the pellet. The lysate was centrifuged at 16,000 x g for 45 min. This and all subsequent steps were carried out at 4° or on ice. Ten ml of 11% streptomycin sulfate was added to each 100 ml of initial supernatant fluid with stirring. Then the resulting suspension was centrifuged at 16,000 x g for 15 min. The supernatant fluid was pooled, and 30 g of solid ammonium sulfate per 100 ml was added slowly with stirring. This precipitate was centrifuged at 16,000 x g for 15 min. The supernatant fluid was pooled, and additional ammonium sulfate was slowly added to a final concentration of 40 g per 100 ml of streptomycin-supernatant fluid. The solution was centrifuged at 16,000 x g for 15 min. The resulting pellet was resuspended in 50 mM KPi buffer and dialyzed against 2 liters of 50 mM KPi buffer overnight.

The dialyzed fraction was injected onto a 21.5 x 150 mm Spherogel-TSK DEAE 5PW HPLC column. The maximum injection contained 160 mg protein. Solvents used were A) 10 mM Tris (pH 7.0) and B) 10 mM Tris (pH 7.0) plus 1 M NaCl. The enzyme was eluted by a series of linear gradients: 5-min wash at 0% B, 5-min gradient to 15% B, 40-min gradient to 31% B, 15-min gradient to 100% B, 15-min wash at 100% B, and a 20-min gradient back to 0% B. The column was then washed for 80 min before another injection was made. One ml fractions were collected. Under these conditions, the enzyme activity

eluted at approximately 79-85 min after the start of the gradient. It should be noted when comparing the retention time to the gradient program that the void volume of the column is approximately 38 ml. The pooled fractions were dialyzed against 1 liter of 150 mM KPi buffer.

The HPLC purified enzyme was then applied to a 1.5 x 40 cm Phenyl-Sepharose CL-4B column equilibrated in 150 mM KPi buffer. The enzyme was eluted from this column by a 500-ml linear gradient from 150 mM to 50 mM KPi buffer, and 6.5-ml fractions were collected. Fractions with the highest specific activity were pooled and precipitated by dialysis against saturated ammonium sulfate. The enzyme was centrifuged at 16,000 x g for 15 min. The pellet was resuspended in a minimal volume of 50 mM KPi buffer and dialyzed against 1 liter of 50 mM KPi buffer. The enzyme was found to be pure by SDS polyacrylamide gel electrophoresis.

Other methods SDS polyacrylamide gel electrophoresis system is that of Ornstein (24) and Davis (25) as modified by Thalacker and Nilsen-Hamilton (26). The N-terminal sequence analysis was performed on a Applied Biosystems gas-phase sequencer by using the method of Hunkapiller et al. (27), with the exception that 25% aqueous trifluoroacetic acid was used for the conversion reaction.

RESULTS

Adenylosuccinate synthetase overproduction The *E. coli purA* gene is carried on a 3.2-kilobase SspI-KpnI DNA restriction fragment. This DNA, pJS76-19, transforms H882 and H1238, two independent *E. coli purA*$^-$ strains, to adenine prototrophy at a high frequency, suggesting that pJS76 contains the *bona fide purA* gene rather than a suppressor gene. Biochemical measurements of adenylosuccinate synthetase activity (below) confirm this conclusion. On the basis of a protein subunit size of 50,000 daltons, the *purA* structural gene is estimated to occupy about 1350 base pairs of the 3.2-kb fragment.

Utilizing flanking HindIII and BamHI sites in the polylinker of pJS76-19, the *purA*-encoding DNA insert was isolated and ligated into HindIII / BamHI double-cut pMOB45 DNA (pMS204) and HindIII / BamHI double-cut pUC18 DNA (pJS76-18) (see Fig. 1). These constructions were transformed into JM101 for assessment of enzyme overproduction.

Adenylosuccinate synthetase activity, at 40 times the wild-type level, was observed when utilizing the vector, pMS204 (Table I). The degree of overproduction was dependent on the timing of the temperature shift, the duration of growth after the shift, and the composition of the medium. Although we did not measure enzyme levels in the cell-free extract, adenylosuccinate synthetase represents approximately 15% of the total cellular protein (Dr. George H. Reed, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, personal communication). Overproduction of adenylosuccinate synthetase was not observed with either

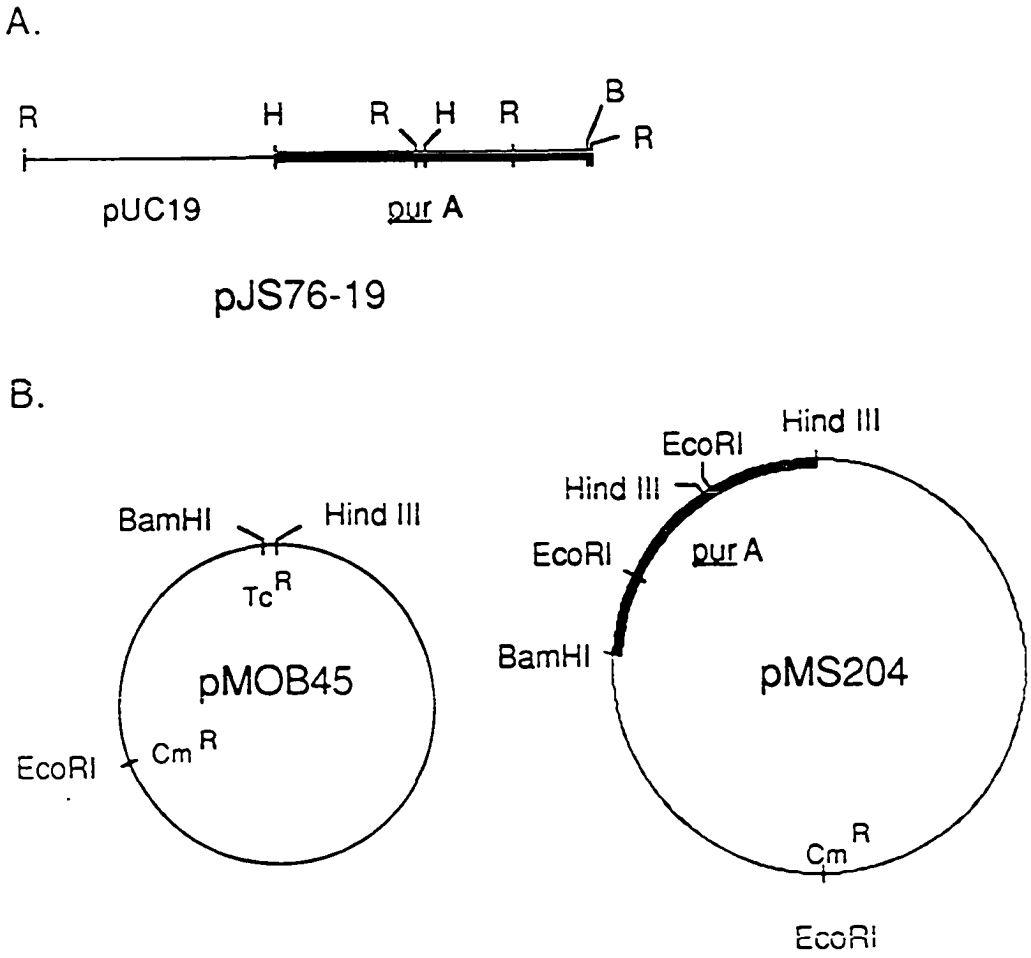

Fig. 1. Restriction maps of pJS76-19, pMOB45, and pMS204, where (A) the plasmid, pJS76-19, is shown linearized at the EcoRI site within the polylinker, where the heavy dark line represents the 3.2-kb, purA-encoding E. coli DNA restriction fragment; and (B) pMS204 is derived from the insertion of the 3.2-kb purA DNA (as a HindIII-BamHI restriction fragment) into HindIII / BamHI double-cut pMOB45 DNA (abbreviations: Cm^R, chloramphenicol resistance locus; Tc^R, tetracycline resistance locus; B, BamHI; H, HindIII; R, EcoRI)

Table I
Growth Conditions for Overproduction of Adenylosuccinate Synthetase[a]

Conditions	Culture Stage[c]	Specific Activity[b] Wild-type[d]	Specific Activity[b] Overproduced	Ratio
pUC Vectors				
pJS76-18	0.2	1.17	5.83	5.0
pJS76-19	0.2	1.17	5.83	5.0
pJS76-18 + IPTG[e]	1.0	3.47	3.71	1.1
pJS76-19 + IPTG	1.0	3.47	3.87	1.1
pMS204 Vector				
Temp Shift[f]	1.2	4.36	126.6	23.8
Temp Shift at OD = 0.7	1.2	5.46	53.3	9.8
30 min Temp Shift[g]	1.0	5.71	102.4	17.9
Temp Shift, Glucose once[h]	1.7	4.05	157.3	38.8
Temp Shift, Glucose twice[i]	2.4	3.51	70.5	20.1

[a]Growth conditions for the expression vectors were 500 ml LB cultures in 2-liter flasks. pUC expression vectors were grown at 37°. pMS204 was grown at 28° until indicated. All ODs were measured at 595 nm.

[b]Measured on an ammonium sulfate fraction of the culture.

[c]OD_{595} at the time of harvest.

[d]Measured on a culture of JM101 grown under the same conditions as the expression vector.

[e]IPTG was added when the cell culture reached OD = 0.2.

[f]Temperature shift to 42° occurred at OD = 0.1 unless otherwise noted.

[g]Temperature shift to 42° for 30 min, then the temperature was reduced to 37°.

[h]Glucose was added to a final concentration of 0.5% when the culture reached OD = 1.0.

[i]The second glucose addition (same amount) occurred when the culture reached OD = 2.0.

orientation of the insert in the pUC vectors, regardless of whether or not transcription from the lacZ promotor was induced (Table I). This is consistent with earlier observations from Collison and Fromm (Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, unpublished observations), that enzyme levels are only slightly elevated in JA200 (pLC14-14), a ColE1 hybrid plasmid previously shown to complement purA mutations (28). In addition, both orientations of the SspI-KpnI DNA fragment have been cloned downstream from the lambda P_L promotor in an expression cassette, which also encoded the cI^{857}, temperature-sensitive lambda repressor protein. Again, no enzyme overproduction could be induced (Dong, Q., Fromm, H. J., and Smith, J. M., Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, unpublished observations).

Purification of adenylosuccinate synthetase Adenylosuccinate synthetase was purified as described in the Experimental Procedures section. Fig. 2 shows a typical elution profile from the DEAE-HPLC column. In comparison to the elution profile obtained when the enzyme is purified from wild-type cells (data not shown), the activity elutes in the shoulder of a nearby peak. The overproduction is quite dramatic in this comparison.

Fig. 3 depicts the elution profile from Phenyl-Sepharose. As can be seen, the activity coelutes with the protein peak. The contaminating proteins elute in the initial wash which can be seen in the beginning of the chromatogram. The purification from 76 g of JM101 (pMS204) cell paste is summarized in Table II. In subsequent purifications, we have obtained as much as 75 mg of pure adenylosuccinate synthetase from the same amount

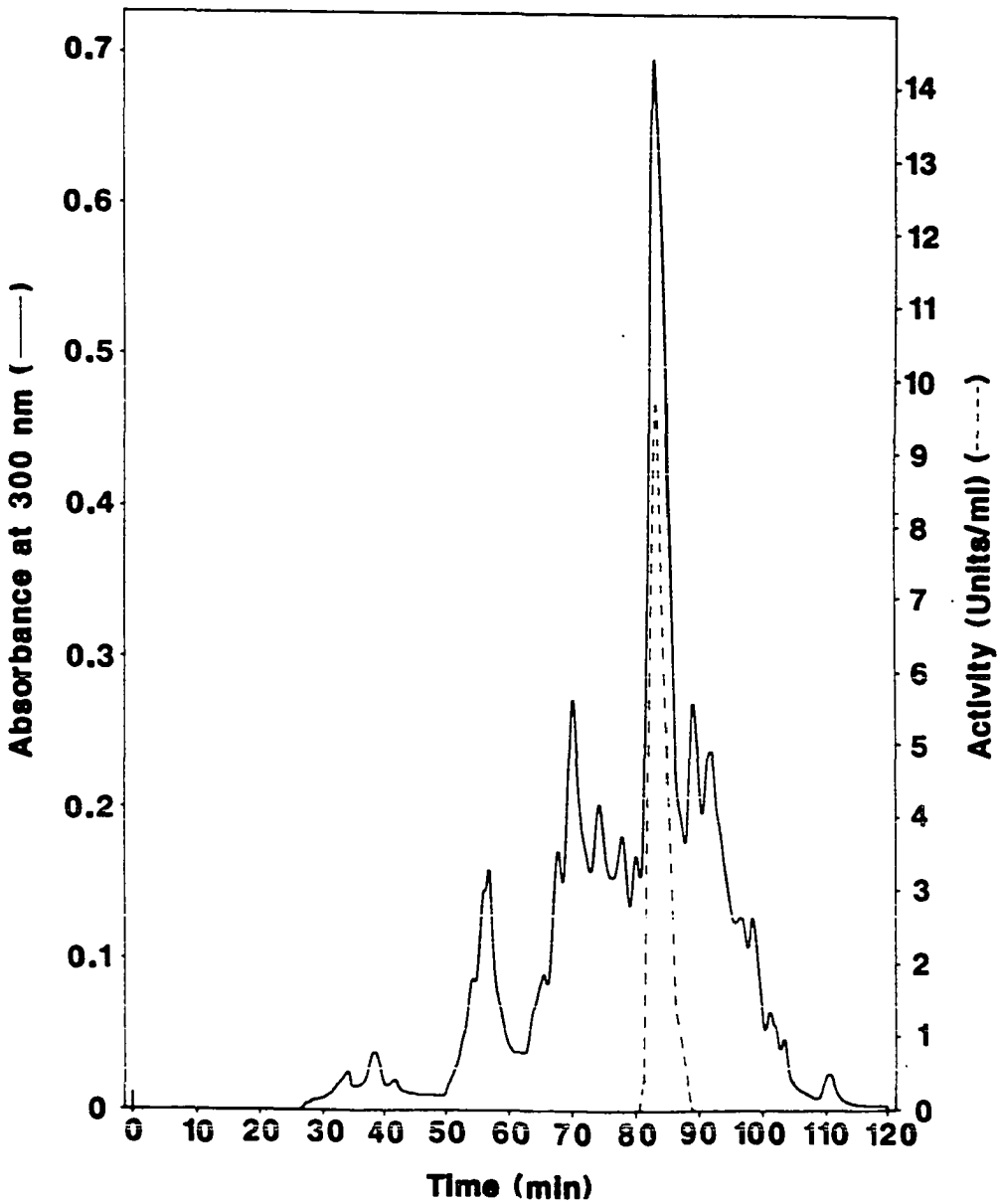

Fig. 2. Elution profile from chromatography of 85 mg of the ammonium sulfate fraction on a 21.5 x 150 mm Spherogel-TSK DEAE 5PW HPLC column, where the solid line represents absorbance at 300 nm and the dashed line represents adenylosuccinate synthetase activity

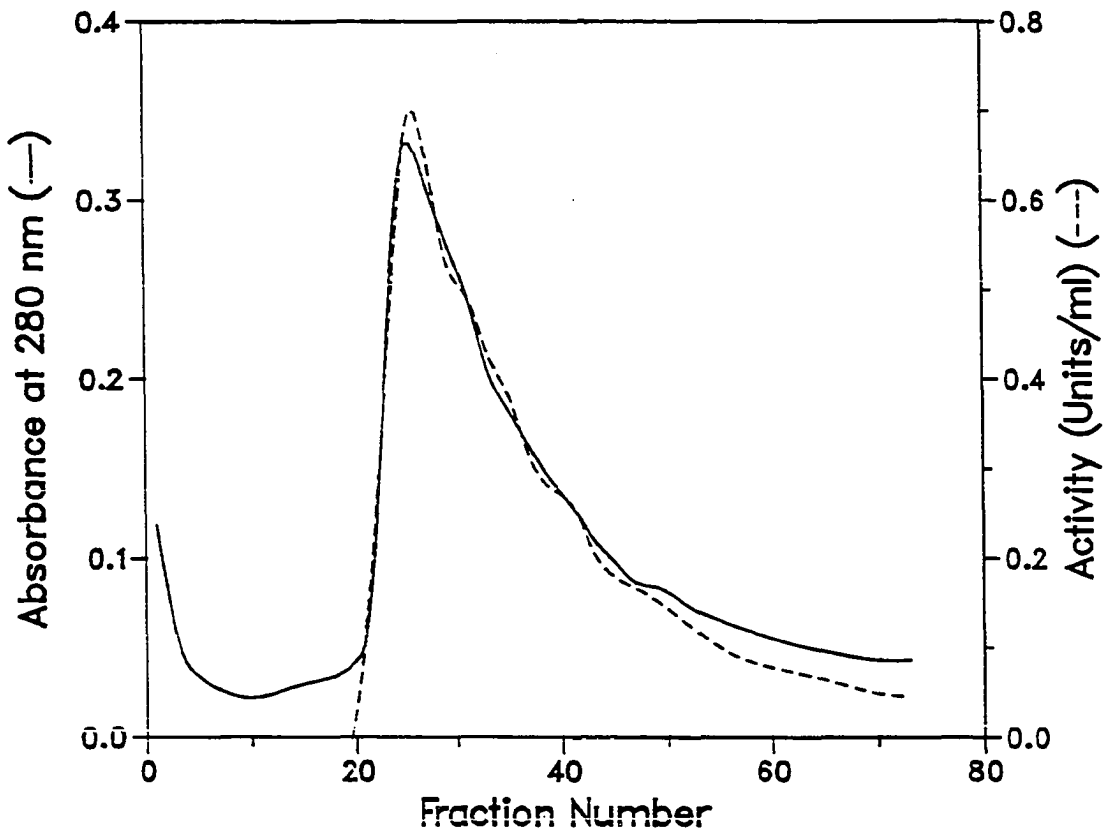

Fig. 3. Elution profile from chromatography of the HPLC purified fraction on a 1.5 x 40 cm column of phenyl-Sepharose, where the solid line represents absorbance at 280 nm and the dashed line represents adenylosuccinate synthetase activity

Table II
Purification Summary of Adenylosuccinate Synthetase[a]

Step	Protein (mg/ml)	Activity (mU/ml)	Specific Activity (mU/mg)	Total Activity (Units)	Purification (fold)
Initial	8.64	1971.	228.	273.	1.0
Streptomycin	6.61	1322.	200.	171.	0.9
Ammonium Sulfate	29.6	11680.	395.	95.8	1.7
DEAE-HPLC	3.90	4137.	1061.	70.3	4.7
Phenyl-Sepharose	11.0	19040.	1731.	41.1	7.6

[a]This table represents the purification from 76 g of JM101 (pMS204) cell paste grown under the conditions described in the text.

of cell paste.

Comparison of wild-type and overproduced enzyme preparations We purified both the wild-type and overproduced proteins by the same procedure and subjected both to tests to verify that the proteins are the same. To get the wild-type protein pure enough for these tests, it was necessary to purify it by a second DEAE HPLC step after the phenyl-sepharose column step. For this step, a 7.5 by 75 mm Spherogel-TSK DEAE 5PW HPLC column was used. Fig. 4 shows a comparison of wild-type and overproduced enzymes on SDS polyacrylamide gel electrophoresis. As can be seen, both proteins have the same molecular weight. Using molecular weight standards, we have determined the subunit molecular weight to be 48,000. This is in good agreement with the subunit molecular weight (47,300) calculated from the gene sequence (Dr. John M. Smith, Louisiana State University, personal communication). To determine the native molecular weight, we used size-exclusion chromatography. Using a 7.5 x 300 mm Spherogel-TSK 3000 SW HPLC gel filtration column, we have shown that both the overproduced and wild-type proteins elute with the same retention time and have an apparent molecular weight of 141,000. Fig. 5 illustrates the standard curve for the molecular weight determination. Cross-linking the protein with dimethyl suberimidate (29) and analysis by SDS-PAGE showed that the native protein is made up of two subunits (data not shown). Therefore, we believe the larger apparent molecular weight by gel filtration is due to the asymmetry of the native protein dimer.

The N-terminal amino acid sequence of both enzyme preparations was

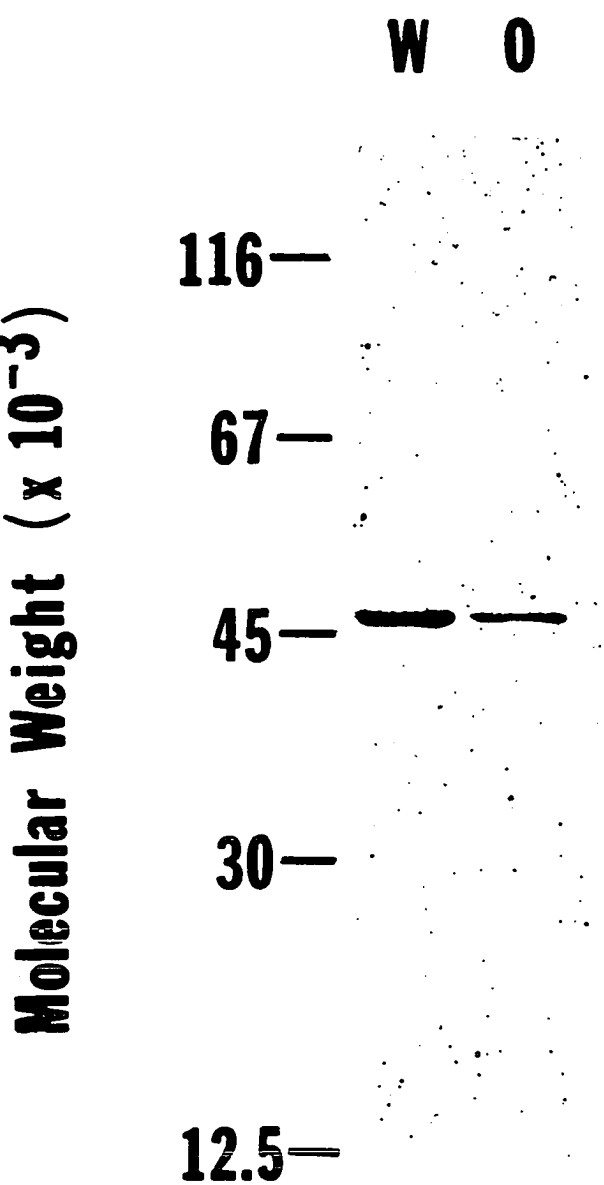

Fig. 4. A picture of the SDS polyacrylamide gel showing wild-type (W) and overproduced (O) adenylosuccinate synthetases

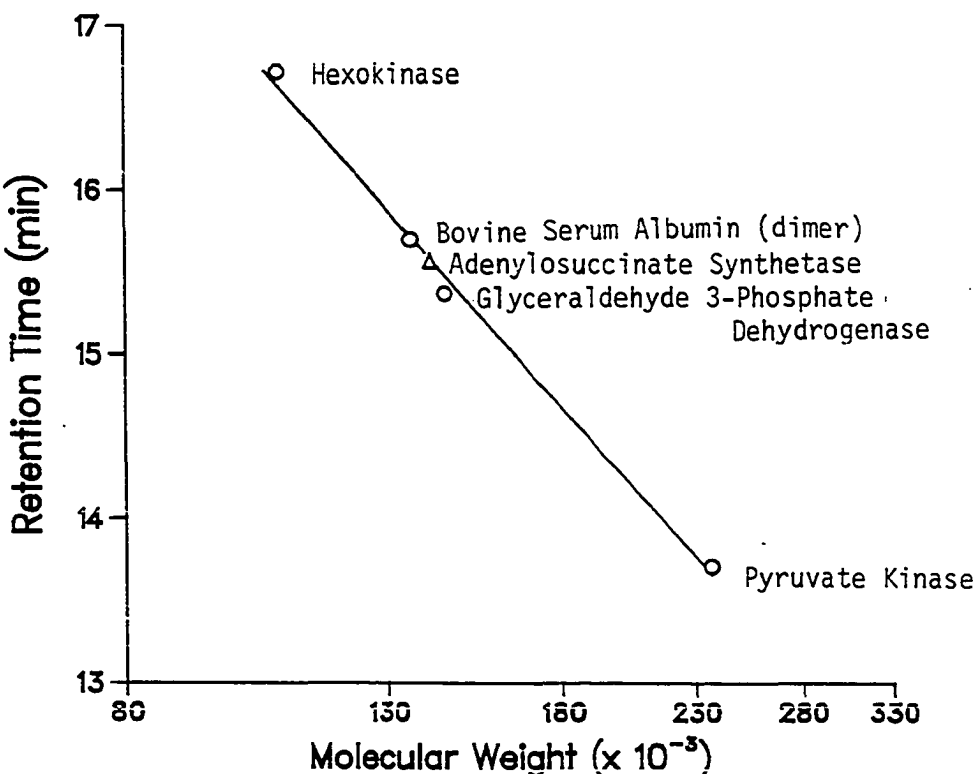

Fig. 5. A plot of the retention time versus the log of the molecular weight

Table III
N-Terminal Sequence Comparison of Adenylosuccinate Synthetases

purA gene[a]	Met-Gly-Asn-Asn-Val-Val-Val-Leu-Gly-Thr-Gln
Overproduced	Gly-Asn-Asn-Val-Val-Val-Leu-Gly-Thr-Gln
Wild-type	Gly-Asn-Asn-Val-Val-Val-Leu-Gly-Thr-Gln

[a]Dr. John M. Smith, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana, personal communication.

determined. We found both sequences to be the same (Table III). The sequence is also the same as that predicted from the purA gene sequence (Dr. John M. Smith, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana, personal communication).

The kinetic parameters of the overproduced enzyme were determined by varying one substrate while holding the other two substrates at near saturating concentrations (30). We report the apparent Michaelis constants for the substrates to be 38 μM for GTP, 54 μM for IMP, and 980 μM for L-aspartate. These values agree with the values reported previously by other investigators (1).

DISCUSSION

The temperature-inducible, high-copy-number vector, pMOB45, was chosen as the vehicle for overproduction of adenylosuccinate synthetase. This vector is characterized by low copy number during cell growth at 30°, changing to runaway plasmid DNA replication upon a temperature shift to 42°. Enzymes such as the dnaC gene product, which is difficult to overproduce by other strategies, was produced at up to 150 times its wild-type levels by use of pMOB45 (31).

E. coli adenylosuccinate synthetase proved unexpectedly resistant to overproduction. The purA gene was not measurably overexpressed in pUC18/19, ColE1, or expression vectors based on the lambda P_L promotor. The 3.2-kb SspI-KpnI DNA fragment carries both the intact purA structural gene and its promotor and operator sequences (Dr. John M. Smith, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana, personal communication). Thus, failure to observe adenylosuccinate synthetase overproduction in relatively low-copy-number vectors may be due to a repression of transcription by ATP and the pur gene repressor protein(s). Consistent with this hypothesis is the observation that the purA gene is subject to adenine repression and, in a manner analogous to the purE gene, probably is partially repressed even in purine-free medium. When cells are grown in 50 µg/ml adenine, adenylosuccinate synthetase activity cannot be detected. Successful overproduction of the enzyme by utilizing a temperature-inducible, runaway replication plasmid suggests that high levels of expression are dependent

on titrating out the repressor activity and thus relieving the inhibition of purA transcription. Further increases in the degree of overproduction may be possible by removing the purA promotor and driving expression of the coding region with a strong, heterologous E. coli or phage promotor.

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SECTION IV: IS RAT SKELETAL MUSCLE HEXOKINASE AN ALLOSTERIC ENZYME?

Is Rat Skeletal Muscle Hexokinase an Allosteric Enzyme?

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ABSTRACT

A recent report by Gregoriou et al. [Eur. J. Biochem., 161, 171-176 (1986)] showed that the mechanism for rat skeletal muscle hexokinase contains two allosteric sites: one for ATP and one for glucose 6-phosphate. In this report, we will show that the allosteric mechanism is at variance with a large amount of kinetic data for the skeletal muscle hexokinase reaction in the literature. In addition, the allosteric mechanism conflicts with isotope exchange at chemical equilibrium data reported by Gregoriou et al. [Eur. J. Biochem. 134, 283-288 (1983)].

INTRODUCTION

In 1983, Gregoriou et al. (1) reported kinetic studies on rat skeletal muscle hexokinase (hexokinase II) on the basis of which they proposed that ATP and glucose 6-phosphate add to allosteric sites on the enzyme. These conclusions are at variance with investigations by Hanson and Fromm (2) and Lueck and Fromm (3) who had proposed a Random Bi Bi[1] mechanism for the phosphotransferase. Ganson and Fromm (5) have recently carried out initial-rate experiments in the nonphysiological direction with hexokinase II as well as studies involving the isotope exchange at chemical equilibrium technique of Boyer (6). Their investigations are in harmony with earlier mechanistic proposals for hexokinase II (2,3). In addition, they indicated that the allosteric mechanism introduced by Gregoriou et al. (1), not only was at variance with results from their laboratory, but did not accord with initial-rate experiments reported by Grossbard and Schimke (7).

In 1986, Gregoriou et al. (8) published a manuscript in which they took exception to some of the criticisms presented by Ganson and Fromm (5), while at the same time accepting other criticisms of their original mechanism. To circumvent the problems suggested by Ganson and Fromm (5) with their 1983 proposal, they presented a modification of the original mechanism (8), which is represented in Scheme 1.

This mechanism is different from its predecessor in three ways: (1) One of the allosteric complexes is eliminated. (2) Two steps are changed from steady-state to rapid-equilibrium interconversions. And (3) the

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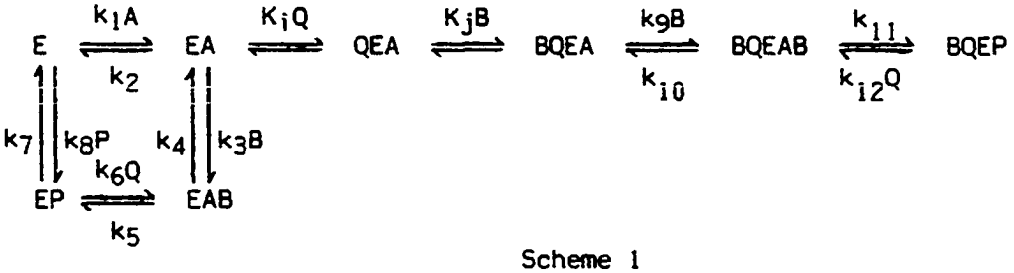
[1]The kinetic nomenclature is that of Cleland (4).

catalytic cycle has ADP, rather than glucose 6-phosphate, dissociating from hexokinase in the only reaction in which free ADP is generated. Gregoriou et al. (8) suggest that the modified mechanism is fully consistent with their flux-ratio data reported earlier (1). In addition, they point out a number of problems associated with the findings of Ganson and Fromm (5) as well as with earlier investigations on skeletal muscle hexokinase from Fromm's laboratory (2,3).

In this report, we respond to the criticisms of Gregoriou et al. (8) regarding our work on hexokinase II. We will show that the new mechanism proposed by these workers is at variance, not only with studies from our laboratory and Schimke's laboratory (7), but from Cornish-Bowden's laboratory (1) as well.

THEORY



Scheme 1

In Scheme 1, E, A, B, P, and Q represent hexokinase, glucose, ATP, ADP, and glucose 6-phosphate, respectively. The model illustrated in Scheme 1 for hexokinase, if not unique, is certainly unusual and therefore requires explanation beyond that provided by Gregoriou et al. (8). The pathway described in Scheme 1 suggests that both glucose and ADP add to the same form of hexokinase, denoted by E in Scheme 1. Because the mechanism is ordered (i.e., sites for ATP and glucose 6-phospnate do not exist before addition of glucose and ADP, respectively, to the enzyme), it must be concluded that two binding sites exist on hexokinase in addition to the allosteric sites. These are glucose and ADP sites. This can be contrasted with, for example, a pyridine-linked anaerobic dehydrogenase, which also has an ordered mechanism but a single site -- a nucleotide binding site. If only one site is assumed to exist on hexokinase (e.g., the glucose binding site), the only site remaining at the end of a single catalytic cycle will be the ADP binding site. The reaction will terminate at this point unless there is an isomerization step that is capable of converting the ADP binding site to a glucose binding site. Because the mechanism

illustrated in Scheme 1 does not provide for an isomerization step after ADP dissociates from hexokinase, we assume that, implicit in the model, is the proposal that two binding sites exist on the enzyme, one for glucose and one for ADP. If this is indeed what the authors of this model propose (8), it can be shown that substrate inhibition by glucose is to be expected as illustrated by the rate expression shown in Equation 1.

$$\frac{E_o}{v} = \frac{1}{k_5} + \frac{1}{k_7}\left(1 + \frac{A}{K_a'}\right) + \frac{1}{k_1(A)} + \frac{k_4 + k_5}{k_3k_5(B)} + \frac{k_2(k_4 + k_5)}{k_1k_3k_5(A)(B)} \qquad (1)$$

The dissociation constant K_a' represents binding of glucose to the E·ADP complex. Equation 1 predicts that glucose will exhibit substrate inhibition (hyperbolic concave-up) in 1/velocity versus 1/glucose plots. To the best of our knowledge, there is no support from initial-rate experiments in the literature to suggest that glucose is a substrate inhibitor of the skeletal muscle hexokinase reaction (2,3,7).

Our laboratory recently carried out isotope exchange studies with hexokinase II to establish its kinetic mechanism (5). The protocol used was originally suggested by Boyer (6) and has been used extensively to study enzyme mechanisms (9). We found that the glucose ⇌ glucose 6-phosphate and ADP ⇌ ATP exchanges increase hyperbolically as the concentration of all substrates, which are held in an equilibrium ratio, increases. These studies provide strong support for the Random Bi Bi mechanism that we proposed for hexokinase II (2,3).

We have derived the isotope exchange expression at chemical equilibrium for that ADP ⇌ ATP exchange using the mechanism of Scheme 1. The equation of the exchange rate, v*, is shown in Equation 2.

$$v^* = \frac{k_4k_6k_8(P)(E_o)}{\left[\frac{k_7(k_4+k_5)}{Q} + k_4k_6\right]\left[1 + \frac{A}{K_{ia}} + \frac{P}{K_{ip}} + \frac{(A)(B)}{K_{ia}K_b} + \frac{(A)(Q)}{K_{ia}K_i} + \frac{(A)(B)(Q)}{K_{ia}K_iK_j} + \right.}$$

$$\left. \frac{k_9(A)(B)^2(Q)}{k_{10}K_{ia}K_iK_j} + \frac{k_9k_{11}(A)(B)^2}{k_{10}k_{12}K_{ia}K_iK_j}\right] \quad (2)$$

Equation 2 predicts that, at very high levels of glucose and glucose 6-phosphate, the ADP ⇌ ATP exchange will be markedly depressed. It is of interest that Gregoriou et al. (1) carried out isotope exchange at equilibrium studies with hexokinase II that bear on the predictions of Equation 2. They report in the first paragraph of the "RESULTS" section of (1): "At saturating concentrations of substrates and products, present at chemical equilibrium, the rates of isotope exchange for ATP ⇌ ADP, ATP ⇌ glucose 6-phosphate, and glucose ⇌ glucose 6-phosphate were very similar." Clearly, these findings are in harmony with our results (5) and serve to exclude the mechanism shown in Scheme 1.

We recently carried out initial-rate experiments with hexokinase II in the nonphysiological direction (5). These experiments, in the absence of inhibitors, were used to exclude the mechanism originally proposed by Gregoriou et al. (1). Gregoriou et al. (8) now suggest that the data shown in Fig. 1 of (5) exhibits "striking curvature visible in all five plots." We wish to respond to this comment by saying that the lines were computer-tested (10) and give excellent fits to a linear model. Nevertheless, because of the criticisms of Gregoriou et al. (8), we derived the initial-

rate equation for the model shown in Scheme 1 to see whether the data in Figs. 1 and 2 of (5) are consistent with this model. The rate expression is described by Equation 3.

$$\frac{Eo}{v} = \frac{1}{k_2}\left(1 + \frac{Q}{K_i}\right) + \frac{1}{k_4} + \frac{1}{k_8(P)} + \frac{(k_4 + k_5)}{k_4k_6(Q)} + \frac{k_7(k_4 + k_5)}{k_4k_6k_8(P)(Q)} \qquad (3)$$

Theoretical plots based on Equation 3 are shown in Figs. 1 and 2. These plots are based on values of the constants from the work of Ganson and Fromm (5) and Gregoriou et al. (8). The only constants that do not have an explicit value are k_2 and k_4. The ratio $k_4/(k_2 + k_4)$ has a range of values from 0 to 1. When $k_4 >> k_2$, the equation predicts that the 1/velocity versus 1/ADP plot will show decreasing slopes as the concentration of glucose 6-phosphate rises. The intercepts, however, will decrease as glucose 6-phosphate is raised at low glucose 6-phosphate, but increase as glucose 6-phosphate is raised at high glucose 6-phosphate. This equation also predicts the 1/velocity versus 1/glucose 6-phosphate plot to be hyperbolic concave-up. The plots shown in Figs. 1 and 2 show the case where $k_2 = k_4$. Here, the increasing intercepts in Fig. 1 at high glucose 6-phosphate, as well as the curvature in the 1/velocity versus 1/glucose 6-phosphate plot (Fig. 2), are still evident. There is not the slightest hint of such curvature in Fig. 2 shown in Ganson and Fromm (5). In the case where $k_2 >> k_4$, the plots will appear to be linear over the range tested. However, linearity is achieved only when the ratio is zero or $k_4 = 0$.

Gregoriou et al. (8) have presented a rate equation for the forward hexokinase reaction in the presence of glucose 6-phosphate and in the

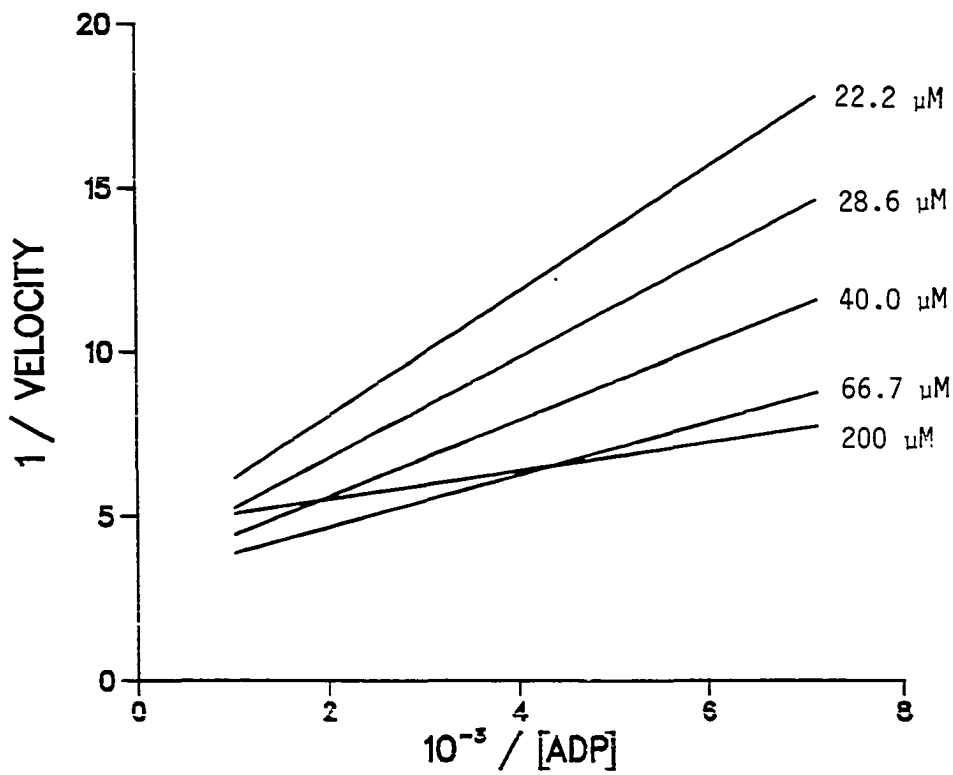


Fig. 1. Plot of the reciprocal of initial velocity versus the reciprocal of the ADP concentration for the hexokinase II reaction in the nonphysiological direction with theoretical lines drawn based on the mechanism shown in Scheme I

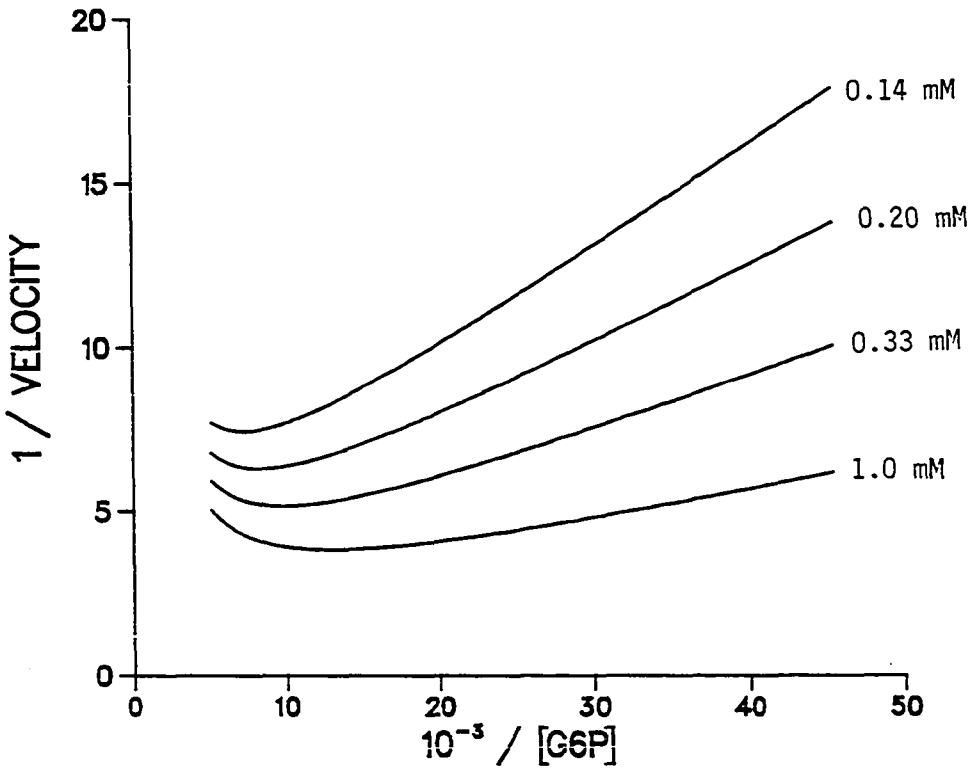


Fig. 2. Plot of the reciprocal of initial velocity versus the reciprocal of the glucose 6-phosphate concentration for the hexokinase II reaction in the nonphysiological direction with theoretical lines drawn based on the mechanism shown in Scheme I

absence of ADP to explain why ATP does not exhibit substrate inhibition, an effect to be expected for the mechanism in Scheme 1. Equation 4 illustrates the product inhibition rate expression.

$$\frac{E_o}{v} = \frac{1}{k_5} + \frac{1}{k_7} + \frac{k_6(Q)}{k_5k_7} + \frac{1}{k_1A} + \left[1 + \frac{Q}{K_i} + \frac{(B)(Q)}{K_iK_j} + \frac{k_9(B)^2(Q)}{k_{10}K_iK_j} + \frac{k_9k_{11}(B)^2}{k_{10}k_{12}K_iK_j}\right]$$

$$\left[\frac{(k_4 + k_5)}{k_3k_5(B)} + \frac{k_4k_6(Q)}{k_3k_5k_7(B)}\right] + \frac{k_2(k_4 + k_5)}{k_1k_3k_5k_7(A)(B)}\left[k_7 + \frac{k_4k_6(Q)}{(k_4 + k_5)}\right] \quad (4)$$

Inspection of the analogous equation by Gregoriou et al. (8) shows that those authors have omitted the $1/k_7$ and all $k_6(Q)/k_5k_7$ terms in their rate equation. They explicitly state that $k_7 >> k_5$ and it is therefore reasonable for them to eliminate the second term in Equation 4; however, no rationale is provided for the elimination of the $k_6(Q)/k_5k_7$ terms. We must therefore assume that the elimination of the $k_6(Q)/k_5k_7$ terms was either an oversight or, alternatively, that k_7 completely dominates the $k_6(Q)/k_5k_7$ terms. If the second supposition is true, then at any finite value of Q, regardless of its magnitude, k_7 would have to approach infinity to eliminate the $k_6(Q)/k_5k_7$ terms. One other possibility to explain the discrepancy in the rate equations would be to assume k_6 approaches zero. Unfortunately, the assumptions that $k_6 \rightarrow 0$ or that $k_7 \rightarrow \infty$ imply in Equation 3 that the velocity of the reaction in the nonphysiological direction is zero. Both Ganson and Fromm (5) and the isotope exchange experiments of Gregoriou et al. (1,8) exclude this possibility. We therefore question the assumptions made by Gregoriou et al. (8) that allow them to omit terms in their rate expression. It is our contention that their model does indeed predict substrate inhibition by ATP, a prediction

that is not borne out in the literature (2,3,7). Furthermore, if the $k_6(Q)/k_5k_7$ terms in Equation 4 are not eliminated, inhibition of glucose 6-phosphate relative to ATP would be nonlinear and not competitive (see Fig. 2 of (8)).

SUMMARY

We can summarize our views on the mechanism of rat skeletal muscle hexokinase as proposed by Gregoriou et al. (8) by stating that their model is untenable, not only on kinetic grounds, but also from the perspective of enzyme chemistry. We are not aware of any enzyme that requires separate substrate and product binding sites in an ordered mechanism, nor are we aware of any enzyme that binds substrates and releases products in the sequence described by the model proposed by Gregoriou et al. (8) for rat muscle hexokinase.

The mechanism proposed in Scheme 1 is at variance, not only with work done in our laboratory (2,3,5) and in Schimke's laboratory (7), but it does not accord with isotope exchange studies at chemical equilibrium reported by Gregoriou et al. (1). We acknowledge that Gregoriou et al. (1) were measuring initial rates of isotope exchange in their flux-ratio experiments (see Fig. 1 of (8)); however, results of this type are to be expected whether the system is at, near, or displaced from chemical equilibrium. If the former conditions prevail, the flux-ratio equations are rendered invalid. Gregoriou et al. (8) insist that their apparent equilibrium constant at pH 6.5 of 234 is correct. We have suggested that if the value is 490 as reported by Robbins and Boyer (11), then the system of Gregoriou et al. (1) will achieve chemical equilibrium with as little as 0.2% product generation (5). We have found data in the literature that indicate that the equilibrium constant for hexokinase at pH 6.5 may be greater than 490[2]

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[2]Dr. Richard Veech, National Institutes of Alcoholism and Alcohol Abuse, St. Elizabeth's Hospital, Washington, DC, in a personal communication reported an equilibrium constant of 1990 at pH 7.0, 1 mM free

(12,13), and we have found data to suggest the value is somewhat below 490 but above the value of 234 suggested by Gregoriou et al. (1,8). Another point that bears on this problem concerns the possible contamination of glucose 6-phosphate with glucose. Because the levels of ADP/ATP in some of the flux-ratio experiments was 5/1, a 1% contamination of the glucose 6-phosphate by glucose would put the system at chemical equilibrium, if the equilibrium constant is about 500. This point is merely a suggestion on our part in an attempt to explain why the model proposed by Gregoriou et al. (8) is so inconsistent with the large volume of kinetic data available in the literature.

On basis of their flux-ratio experiments, Gregoriou et al. (1,8) have suggested that the Random mechanism originally proposed by Hanson and Fromm (2) is not a viable model for rat muscle hexokinase. The findings of Ganson and Fromm (5) and this report imply that little credence can be placed on the two models for hexokinase II proposed by Cornish-Bowden and his coworkers (1,8). On the other hand, the Random mechanism is supported by a variety of data involving initial-rate studies of the forward and reverse reaction, product and substrate analogue inhibition, and isotope exchange at chemical equilibrium experiments (2,3,5,7). The only data in the literature that seem to be at variance with the Random model are the flux-ratio studies of Gregoriou et al. (1,8). The improbability of the mechanism described in Scheme 1 seems to enhance the random pathway as the most probable mechanism for rat skeletal muscle hexokinase.

Mg^{2+}, and 38°. This computes to a value of 630 at pH 6.5.

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GENERAL SUMMARY

This dissertation has shown the reaction mechanism for the adenylosuccinate synthetase reaction occurs via a 6-phosphoryl-IMP intermediate as originally proposed by Lieberman (2). A recent report by Cooper et al. (14) used the technique of isotope exchange at equilibrium to investigate the kinetic mechanism of adenylosuccinate synthetase from rat muscle. They found the reaction is indeed Random Ter Ter but that there is a preferred order for addition of reactants with aspartate adding to an enzyme-GTP-IMP complex. This finding supports the formation of a 6-phosphoryl-IMP intermediate, since the enzyme favors GTP and IMP binding before aspartate. Because no partial exchange reaction occurs (3,14), the extent of intermediate formation is undoubtedly limited.

Overproduction and purification of the adenylosuccinate synthetase from E. coli was shown using a runaway-replication plasmid. This allows large quantities of homogeneous enzyme to be purified. Obtaining large quantities of the E. coli enzyme will further studies on the characterization of the enzyme. Currently several laboratories including this one are utilizing the overproduced enzyme to study enzymatic properties and determine the three-dimensional structure of the protein.

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